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Contribution of the N-Terminal and C-Terminal Domains of Haemaphysalin to Inhibition of Activation of Plasma Kallikrein-Kinin System

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Haemaphysalin is a kallikrein-kinin system inhibitor from hard tick *Haemaphysalis longicornis*, and consists of two Kunitz type protease inhibitor domains. Each domain as well as haemaphysalin inhibited intrinsic coagulation by inhibiting activation of the kallikrein-kinin system without affecting the amidolytic activities of intrinsic coagulation factors, indicating that both domains were involved in the inhibition through a similar mechanism to that for haemaphysalin. Reconstitution experiments showed that the C-terminal domain contributed more predominantly to this inhibition. Direct binding assaying showed that the C-terminal domain could bind to the cell-binding region of high molecular weight kininogen (HK), suggesting that it also binds to the cell-binding region of factor XII. Judging from these findings, the C-terminal domain may more effectively inhibit the association of factor XII and HK with the cell surface by binding to cell-binding regions, and hence would predominantly contribute to the inhibition of activation of the kallikrein-kinin system.

Key words: kallikrein-kinin system, Kunitz type protease inhibitor, tick.

Abbreviation: HK, high molecular weight kininogen; CTI, corn trypsin inhibitor; DS500, dextran sulfate of MW 500,000; SBTI, soybean trypsin inhibitor; S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide; S-2302, H-D-Pro-Phe-Arg-*p*-nitroanilide; S-2366, pyro-Glu-Pro-Arg-*p*-nitroanilide; Spectrozyme® fIXa, $MeSO_2$-D-CHG-Gly-Arg-*p*-nitroanilide; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide; GST, gluthathione S-transferase; RP-HPLC, reverse phase HPLC; APTT, activated partial thromboplastin time; SPR, surface plasmon resonance; RU, resonance unit.

The kallikrein-kinin system in human plasma principally consists of three plasma proteins, factor XII, prekallikrein, and HK. Factor XII and the prekallikrein-HK complex assembles on a biologic activating surface or a negatively charged surface, and activation of the kallikrein-kinin system occurs. *In vivo*, the binding of the prekallikrein-HK complex to a biologic activating surface, such as endothelial cells, initiates activation of the kallikrein-kinin system (*1*). On the cell surface, prekallikrein is converted to kallikrein by prolylcarboxypeptidase, and the kallikrein generated converts factor XII to factor XIIa (*2–4*). *In vitro*, the binding of factor XII to a negatively charged surface initiates activation of the kallikrein-kinin system. On the negatively charged surface, factor XII is autoactivated to factor XIIa, which catalyzes prekallikrein activation.

Activation of the kallikrein-kinin system is followed by reciprocal activation of factor XII and prekallikrein, and this accelerates the generation of factor XIIa and kallikrein (*5*). The kallikrein generated cleaves HK, releasing bradykinin, a primary mediator of the inflammation. Bradykinin induces symptoms such as redness, edema, and pain around the injured site (*6*). On the other hand, the factor XIIa generated initiates the intrinsic coagulation pathway by activating factor XI. However, the physiologic significance of this pathway is questionable, since deficiencies of factor XII, prekallikrein, and HK are not associated with hemorrahagic diathesis (*7*). Therefore, the plasma kallikrein-kinin system may play an important role in the initiation of acute-inflammatory responses.

Zn^{2+} ions are involved in the binding of factor XII and HK to a biologic activating surface (*8–11*). The binding of Zn^{2+} ions to these molecules induces conformational changes in them (*12–14*). These conformational changes are essential for the binding of factor XII and HK to the biologic activating surface (*15–17*). The fibronectin type II domain and domain D5 act as cell-binding domains of factor XII and HK, respectively, and factor XII and HK interact with the biologic activating surface through these cell-binding domains (*18*, *19*). In addition, factor XII and HK compete for the biologic activating surface, indicating that there is a putative common receptor on the surface, and factor XII and HK interact with this receptor via each cell binding domain (*15–17*).

Blood sucking arthropods, such as mosquitoes, flies, kissing bugs, and ticks, have various pharmacologically active compounds in their saliva (*20–22*). These com-

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pounds allow arthropods to feed on blood from host animals (*23*). Namely, these compounds are injected into the host animals when arthropods feed on blood, and inhibit host defense responses, such as coagulation, vasoconstriction, and immune responses. Various pharmacological active compounds, such as anticoagulant molecules (*24*, *25*), platelet aggregation inhibitors (*26*, *27*), vasodilators (*28*, *29*), and immunosuppressive proteins (*30*), had been identified in the saliva and/or salivary glands of these arthropods. Recently, in order to identify novel pharmacological active molecules, extensive analyses of cDNA expressed in salivary glands of blood sucking arthropods were performed (*31*–*33*). These analyses indicated that there are various pharmacological active compounds (*34*, *35*).

Earlier, we performed a mass sequence analysis of salivary gland cDNA from the hard tick *Heamaphysalis longicornis*, and identified a unique inhibitor of the kallikrein-kinin system (*36*). This inhibitor, named haemaphysalin, consists of two Kunitz type protease inhibitor domains, and inhibits the intrinsic coagulation pathway without affecting the amidolytic activities of any intrinsic coagulation factors. We further found that haemaphysalin inhibited activation of the kallikrein-kinin system by interfering with all steps of reciprocal activation of factor XII and prekallikrein. This finding indicated that haemaphysalin inhibited intrinsic coagulation by interfering with activation of the kallikrein-kinin system. On the other hand, we showed that haemaphysalin could bind specifically to factor XII/XIIa and HK, and also bound to their cell-binding domains, the fibronectin type II domain of factor XII and domain D5 of HK. Therefore, we suggested that haemaphysalin interferes with factor XII and HK binding to a biologic activating surface by binding to their cell-binding regions, thereby inhibiting their reciprocal activation on the surface.

In the present study, we investigated the contribution of each Kunitz type protein domain of haemaphysalin to inhibition of the kallikrein-kinin system activation. It was strongly suggested that both domains as well as haemaphysalin could inhibit the intrinsic coagulation without inhibiting the amydolytic activities of any intrinsic coagulation factors. We further showed that both domains inhibited activation of the kallikrein-kinin system by inhibiting reciprocal activation. A direct binding assay showed that the C-terminal domain exerted stronger inhibitory activity toward all steps of reciprocal activation than the N-terminal domain, indicating that the C-terminal domain predominantly contributed to the inhibition of activation of the kallikrein-kinin system. On the other hand, the C-terminal domain, but not the N-terminal domain, could bind to the cell-binding region of domain D5 of HK, suggesting that it also binds to the cell-binding region of the fibronectin type II domain of factor XII. Therefore, we assumed that the C-terminal domain more effectively inhibited the association of factor XII and HK with a biologic activating surface by binding to their cell-binding regions than the N-terminal domain.

MATERIALS AND METHODS

Materials—Factor IXa, factor Xa, factor XIa, factor XII, factor XIIa, prekallikrein, kallikrein, thrombin, and HK were purchased from Enzyme Research Laboratories (South Bend, IN). To determine protein concentrations, the following extinction coefficients ($E^{1\%}_{280}$) and molecular weights were used: factor IXa, 14.9, 56,000; factor Xa, 11.6, 46,000; factor XIa, 13.1, 160,000; factor XII, 14.1, 80,000; factor XIIa, 14.1, 80,000; prekallikrein, 11.7, 86,000; kallikrein, 11.7, 86,000; thrombin, 18.3, 37,000; and HK, 7.01, 120,000. Citrated human normal plasma was purchased from Bio Mérieux S.A. (Marcy l'Etoile, France). Corn trypsin inhibitor (CTI) was obtained from Calbiochem (San Diego, CA). Dextran sulfate of MW 500,000 (DS500) and soybean trypsin inhibitor (SBTI) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Chromogenic substrates (S-2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide; S-2302, H-D-Pro-Phe-Arg-*p*-nitroanilide; and S-2366, pyro-Glu-Pro-Arg-*p*-nitroanilide) were purchased from Chromogenix AB (Mölndel, Sweden). Spectrozyme® fIXa ($MeSO_2$-D-CHG-Gly-Arg-*p*-nitroanilide) was obtained from American Diagnostica Inc. (Greenwich, CT). Other reagents used in this study were obtained from Wako Pure Chemical Industries, Ltd., or Nacalai Tesque.

Preparation of Recombinant Proteins of the N-Terminal and C-Terminal Domains of Haemaphysalin—Based on the positions of cysteine residues conserved in the Kunitz type protease inhibitor family, haemaphysalin was divided into two domains. The N-terminal and C-terminal domains consisted of Lys23–Arg89 and Asn85–Arg162, respectively (*36*). The DNA fragments encoding the N-terminal and C-terminal domains of haemaphysalin were each amplified by PCR using a pair of specific primers (N-terminal domain: forward primer, 5′-CATATGAAATATCGAGCCAACCCAGCACTC-3′, reverse primer, 5′-GGATCCCTACTTGATTGTTTATTGCACGTTTCTA-3′; and C-terminal domain: forward primer, 5′-CATATGCAATAAACAATCAAGGTGCCTACAG-3′, reverse primer, 5′-GGATCCCTAGCGCGGGTAAGTACCTTGAGC-3′). The amplified DNA fragments were cloned into the *Nde*I–*Hin*dIII sites of expression vector pET22-b (Novagen, Darmstadt, Germany). The constructed expression plasmids were introduced into the *E. coli* BL21(DE3) strain, and then production of recombinant proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside. Recombinant N-terminal and C-terminal domains formed inclusion bodies in the *E. coli* cells. To obtain the inclusion bodies, *E. coli* cells expressing the recombinant proteins were harvested and sonicated in 50 mM Tris-HCl, pH 8.0, containing 15 μM pepstatin A, 15 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. Recombinant proteins from the inclusion bodies were refolded with a thiol-disulfide shuffling system using reduced and oxidized glutathione (*37*). After the refolding reaction, the recombinant proteins were further purified by gel filtration chromatography on Sephadex G-75 equilibrated with Tris-HCl, pH 7.5. To confirm disulfide bond formation, the free SH groups derived from Cys residues in the recombinant proteins were

titrated using a fluorescent reagent, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM). The titration procedure was as described previously (*36*).

Preparation of Recombinant Proteins of Domains and Peptides Derived from Factor XII and High Molecular Weight Kininogen—The recombinant protein of the fibronectin type II domain of factor XII was produced in the *E. coli* BL21 (DE3) codon-plus RIL strain using pET22-b, and further purified by cation exchange chromatography on Mono S HR5/5 (Amersham Bioscience), followed by gel filtration chromatography on TSK2000SW (TOSOH, Japan).

The recombinant domain D5 of HK was expressed in the *E. coli* BL21(DE3) using pET22-b. Purification of recombinant domain D5 was performed according to Herwald *et al*. (*14*). Briefly, the expressed domain D5 was purified by affinity chromatography on Ni-nitriloacetic acid Sepharose (QIAGEN, Hilden, Germany), followed by gel filtration chromatography on TSK2000SW.

Three peptides, HK383–420, HK 421–466, and HK459–513, were named after the amino acid sequence of HK, and produced as fusion proteins with gluthathione S-transferase (GST) using pGEX6P-1 and the *E. coli* BL21 strain. All GST-peptides were purified by affinity chromatography on glutathione Sepharose 4B (Amersham Bioscience), and then cleaved with PreScission™ protease to remove GST according to the manufacturer's instructions (Amersham Bioscience). After cleavage, all peptides were further purified by reverse phase HPLC (RP-HPLC) on a Wakosil 5C4 column (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Assay for Inhibitory Activities of the N-Terminal and C-Termnal Domains toward Coagulation Factors—The inhibitory activities of the N-terminal and C-terminal domains toward coagulation factors, kallikrein, factor XIIa, factor XIa, factor Xa, factor IXa, and thrombin, were assessed using chromogenic substrates. The procedures were described in detail previously (*36*). The chromogenic substrates used in each assay were as follows: S-2302 for the kallikrein or factor XIIa assay; S-2366 for the factor XIa assay; S-2222 for the factor Xa assay; S-2238 for the thrombin assay; and Spectrozyme® fIXa for the factor IXa assay.

Inhibition Assay for Generation of Thrombin in Plasma—To assess the inhibition of intrinsic coagulation, assaying was performed as follows. Normal human plasma was diluted 50-fold with 50 mM Tris-HCl, pH7.5, containing 150 mM NaCl. The diluted plasma (40 μl) and each sample (20 μl) were mixed, and then the kallikrein-kinin system in the plasma was activated by the addition of 20 μl of 100-fold diluted activated partial thromboplastin time (APTT) reagent (DADE BEHRING, Liederbach, Germany). After incubation for 10 min at 37°C, 50 mM $CaCl_2$ (20 μl) was added to the mixture, followed by further incubation for 20 min. The reaction was stopped by the addition of 50 mM EDTA, pH 7.5 (100 μl), and then the activity of the thrombin generated was photometrically measured at 405 nm using chromogenic substrate, S-2238 (*38*).

The inhibition of activation of the kallikrein-kinin system in plasma was also examined. To activate the kallikrein-kinin system in plasma before the addition of a sample, diluted plasma (40 μl) and 100-fold diluted APTT reagent (20 μl) were mixed and preincubated for 10 min at 37°C. Each sample (20 μl) was then added to the mixture, followed by further incubation for 10 min. After incubation, 50 mM $CaCl_2$ (20 μl) was added to the mixture and the generation of thrombin in the plasma was measured as described above.

Inhibition Assay for the Reciprocal Activation of Factor XII and Prekallikrein—The effects of the N- and C-terminal domains of haemaphysalin on reciprocal activation of factor XII and prekallikrein were examined by mean of a reconstitution assay involving purified proteins, factor XII/XIIa and prekallikrein/kallikrein (*5*, *40*). All assays were carried out on 96-microwell plates at room temperature.

To assay the effect on reciprocal activation of factor XII and prekallikrein, factor XII (final concentration, 0.2 nM) was preincubated with a sample for 10 min in reaction buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% BSA, and 0.1% polyethylene glycol 8000). After incubation, DS500 (0.2 μg/ml) and prekallikrein (10 nM) were added to the mixture, and then autoactivation of factor XII and reciprocal activation began. After 10 min incubation, a chromogenic substrate, S-2302 (170 μM), was added to the mixture and then the increase in absorbance at 405 nm was recorded at 5 min intervals.

To assay the effect on activation of factor XII by kallikrein, factor XII (20 nM) was preincubated with a sample for 10 min in reaction buffer. Kallikrein (0.2 nM) and DS500 (0.3 μg/ml) were added to the mixture, and then activation of factor XII was started. After 10 min incubation, SBTI (0.6 μM) and S-2302 (340 μM) were added to the mixture, and then the activity of factor XIIa generated was monitored at 5 min intervals. The SBTI was added to inhibit the kallikrein acitivity in the reaction mixture.

To assay the effect on prekallikrein activation by factor XIIa, factor XIIa (50 pM) was incubated with a sample in reaction buffer. After 10 min incubation, prekallikrein (10 nM) and DS500 (0.1 μg/ml) were added to the mixture, after which prekallikrein activation began. After incubation for 5 min, CTI (100 nM) and S-2302 (170 μM) were added to the mixture, and then the absorbance change was recorded at 5 min intervals, as described above. The CTI was added to avoid factor XIIa activity in the mixture.

Binding Analysis with Surface Plasmon Resonance—Binding of each domain of haemaphysalin to factor XII/XIIa and HK was analyzed at 25°C by surface plasmon resonance (SPR) spectrometry using a BIAcore 3000 instrument (BIAcore AB, Sweden). The HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 0.005% Tween 20) used in the assay was treated with Chelex 100 (BIO-RAD, Hercules, CA) in advance. $ZnCl_2$ was added to metal-chelated HBS buffer before each assay. Recombinant proteins of each domain and target molecules were also treated with Chelex 100 to avoid metal ions contamination. Briefly, they were dialyzed against metal-chelated HBS buffer containing 0.1% Chelex 100.

Each domain of haemaphysalin was immobilized on the surface of a sensor chip CM4 using an amine coupling kit according to the manufacturer's instructions. After immobilization, each target molecule was injected for 2 min at 20 μl/min and then the association was monitored. After a return to buffer flow, dissociation of the sample-

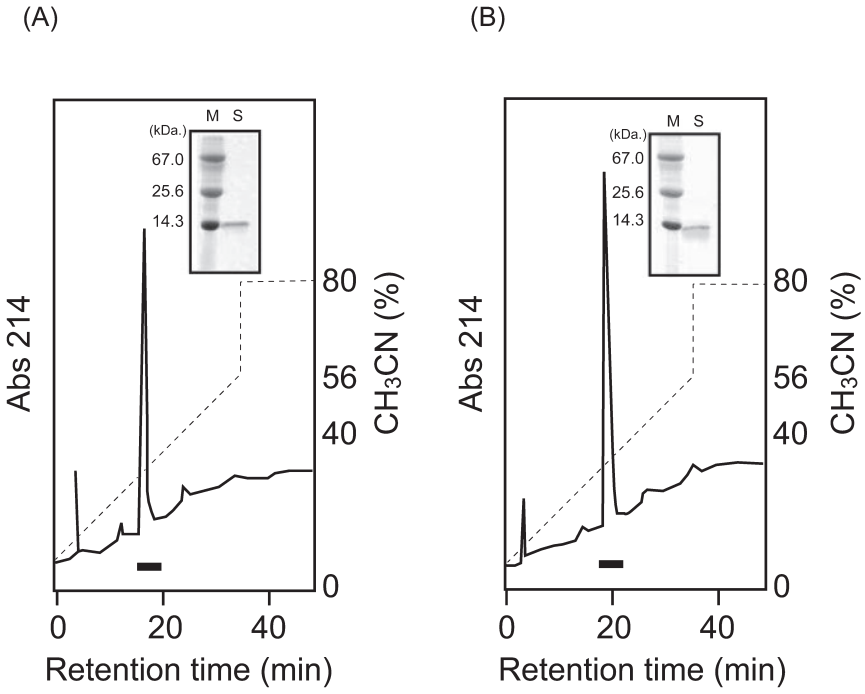

Fig. 1. **Purities of Recombinant proteins of the N-terminal and C-terminal domains.** The purities of the recombinant N-terminal domain (A) and C-terminal domain (B) of haemaphysalin were determined by RP-HPLC and SDS-PAGE. After purification by gel filtration chromatography, the purified recombinant proteins were subjected to RP-HPLC on a Wakosil 5C4 column equilibrated with 0.1% trifluoroacetic acid. The bound proteins were eluted with a gradient of 0 to 80% acetonitrile and 0.1% trifluoroacetic acid. The purified recombinant proteins were also analyzed by SDS-PAGE on a 15% gel, as shown in insets.

target molecule complex was monitored for 2 min. The Zn^{2+} ion dependencies of interactions were examined using HBS buffer containing various concentrations of $ZnCl_2$. Regeneration of the sensor chip surface was achieved by pulse injection of 1 M NaCl and 50 mM EDTA, pH7.4. The exact amounts of immobilized domain on sensor chips are indicated in the figures. The interaction of each domain of haemaphysalin with domains and peptides derived from factor XII and HK was analyzed using a similar procedure.

Kinetic constants were determined from sensorgrams using BIA evaluation software installed in the BIAcore 3000. In the presence of Zn^{2+} ions, a two state binding model was used for the calculation of kinetic constants (k_{a1}, k_{d1}, k_{a2}, and k_{d2}) of the interaction. The kinetic model was as follows:

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\Leftrightarrow}} AB \underset{k_{d2}}{\overset{k_{a2}}{\Leftrightarrow}} (AB)^*$$

In the absence of Zn^{2+} ions, the kinetic constants (K_D, k_a, and k_d) of the interaction between haemaphysalin and factor XIIa well fitted a simple 1:1 Langmuir binding model. The kinetic model was as follows:

$$A + B \underset{k_d}{\overset{k_a}{\Leftrightarrow}} AB$$

RESULTS

Preparation of Recombinant Proteins of the N-Terminal and C-Terminal Domains of Haemaphysalin—Recombinant proteins of the N-terminal and C-terminal domains were produced in *E. coli* cells, harvested as inclusion bodies, and refolded using the disulfide shuffling system developed for the refolding of bovine pancreatic trypsin inhibitor, a well known Kunitz type protease inhibitor (*37*). After the refolding reaction, the recombinant proteins were purified by gel filtration chromatography, and then their purities were confirmed by SDS-PAGE and RP-HPLC analyses (Fig. 1). After purification, disulfide bond formation in the purified recombinant proteins was investigated. Titration assays using a fluorescence reagent, DACM, showed that the purified recombinant proteins did not contain any free Cys residues (Fig. 2). In

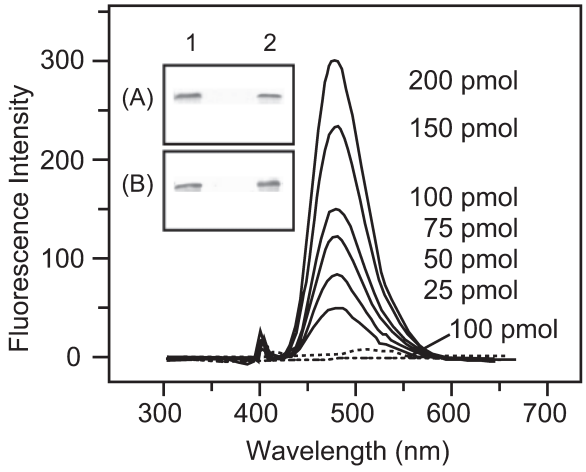

Fig. 2. **Investigation of disulfide bond formation in the recombinant N-terminal and C-terminal domains.** The SH-groups derived from free Cys residues in the recombinant N-terminal and C-terminal domains were titrated using DACM. The solid line (—) indicates the fluorescence intensity of β-mercaptethanol used as a standard. The dashed lines indicates the fluorescence intensities of 100 pM N-terminal domain (– –) and C-terminal domain (–·–). Insets (A) and (B) show SDS-PAGE analyses of the N-terminal and C-terminal domains, respectively. Samples were electrophoresed under reducing (lane 1) and non-reducing conditions (lane 2).

addition, the electrophoretic migration of the purified proteins did not differ under reducing and non-reducing conditions on SDS-PAGE analyses (Fig. 2). Thus, we considered that all Cys residues in the recombinant proteins formed disulfide bonds intramolecularly.

Inhibition by the N-Terminal and C-Terminal Domains of the Intrinsic Coagulation Pathway—In our earlier study, we found that haemaphysalin could inhibit intrinsic coagulation without affecting amidolytic the activities of any intrinsic coagulation factors toward chromogenic substrates. Thus, we first investigated the inhibitory activities of the two domains toward intrinsic coagulation factors (kallikrein, factor XIIa, factor XIa, factor Xa, factor IXa, and thrombin) using appropriate chromogenic substrates. This assay showed that both domains could not inhibit the amidolytic activities of these coagulation factors (data not shown).

Next, we investigated the effects of the two domains on the intrinsic coagulation using human normal plasma. As shown in Fig. 3, both domains inhibited generation of thrombin in plasma activated by APTT reagent. These results showed that both domains could also inhibit the intrinsic coagulation without affecting the amidolytic activities of intrinsic coagulation factors.

We further examined the inhibitory activities of the two domains toward APTT-reagent preactivated plasma. In this condition, the kallikrein-kinin system is already activated before the addition of both domains. In the absence of Ca^{2+} ions, factor XIa was generated but could not activate factor IX, thus preventing downstream reactions. The addition of Ca^{2+} ions allowed the cascade to proceed, but neither domain could inhibit the generation of thrombin, showing that they could not inhibit reactions downstream of the kallikrein-kinin system in plasma (Fig. 3). Therefore, it was suggested that both domains as well as haemaphysalin inhibited the intrinsic coagulation by interfering with the activation of the kallikrein-kinin system.

Effects of the N-Terminal and C-Terminal Domains of Haemaphysalin on Activation of the Kallikrein-Kinin System—Our previous study showed that haemaphysalin inhibits activation of the kallikrein-kinin system by interfering with all steps of reciprocal activation of factor XII and prekallikrein. Thus, we examined the inhibition by the two domains of the reciprocal activation in a reconstitution system. This assay clearly showed that both domains could inhibit the reciprocal activation (Fig. 4A). In addition, both domains also inhibited activation of factor XII and prekallikrein catalyzed by kallikrein and factor XIIa, respectively (Fig. 4, B and C). These results demonstrated that each domain could inhibit all steps of reciprocal activation, indicating that they were involved in the inhibition of activation of the kallikrein-kinin system. Furthermore, it was demonstrated that the C-terminal domain exhibited stronger inhibitory activity than the N-terminal domain in all assays (Fig. 4, A–C). These results demonstrated that the C-terminal domain more predominantly contributed to the inhibition of the kallikrein-kinin system than the N-terminal domain.

Interactions of the N-Terminal and C-Terminal Domains of Haemaphysalin with Factor XII/XIIa—The target molecules of haemaphysalin are factor XII/XIIa and HK. Haemaphysalin recognizes Zn^{2+} ion-induced conformational changes of these molecules and specifically binds to their cell-binding domains. To determine the contributions of the N-terminal and C-terminal domains to the interaction with target molecules, we first examined the interaction of the two domains with factor XII/XIIa in the present work.

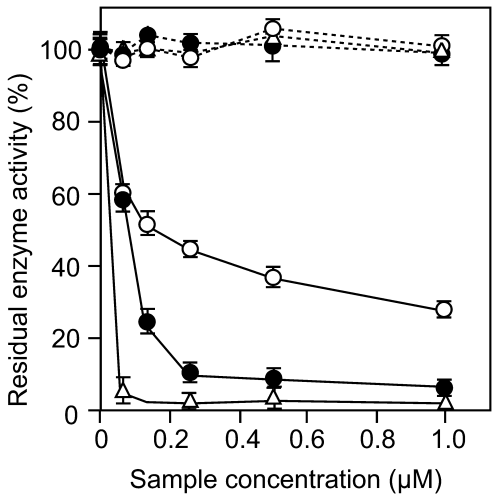

Fig. 3. **Inhibitory effects of the N-terminal and C-terminal domains on thrombin generation in plasma activated by APTT reagent.** The inhibitory activities of haemaphysalin (open triangles), the-N-terminal domain (open circles), and the C-terminal domain (solid circles) toward thrombin generation in human normal plasma were examined (solid lines). In this assay, the kallikrein-kinin system in plasma was activated after mixing of 50-fold diluted plasma and each sample. After activation, $CaCl_2$ was added to the mixture and then downstream cascade reactions started. The reaction was stopped by the addition of EDTA, and then the activity of thrombin generated was photometrically measured at 405 nm using S-2238. The inhibitiory activities of haemaphysalin (open triangles), the N-terminal domain (open circles), and the C-terminal domain (solid circles) toward activation of the kallikrein-kinin system in plasma were also examined (dashed lines). In this assay, the kallikrein-kinin system in plasma was activated before the addition of each sample. The diluted plasma and 100-fold diluted APTT reagent were mixed and preincubated to activate the kallikrein-kinin system. After incubation, each sample was added to the mixture. After activation, $CaCl_2$ was added to the mixture and then the generation of thrombin in plasma was measured, as described above. In all assays, negative control experiments were performed using lysozyme (solid triangles).

Both domains of haemaphysalin interacted with factor XII in the presence of Zn^{2+} ions. In the absence of Zn^{2+} ions, the N-terminal domain, but not the C-terminal domain, could bind to factor XII (Fig. 5, A and B, and Tables 1 and 2). Furthermore, the binding of the N-terminal domain to factor XII gradually increased with the Zn^{2+} ion concentration (Fig. 5A). The C-terminal domain did not interact with factor XII at below 10 μM. The binding response of the C-terminal domain to factor XII increased depending on the Zn^{2+} concentration over 25 μM (Fig. 5B). These results showed that effect of Zn^{2+} ions on the interaction with factor XII differs between the N-terminal and C-terminal domains.

The N-terminal and C-terminal domains could interact with factor XIIa in the presence and absence of Zn^{2+} ions (Fig. 5, C and D, and Tables 1 and 2). These domains bound tightly to factor XIIa with the addition of Zn^{2+} ions,

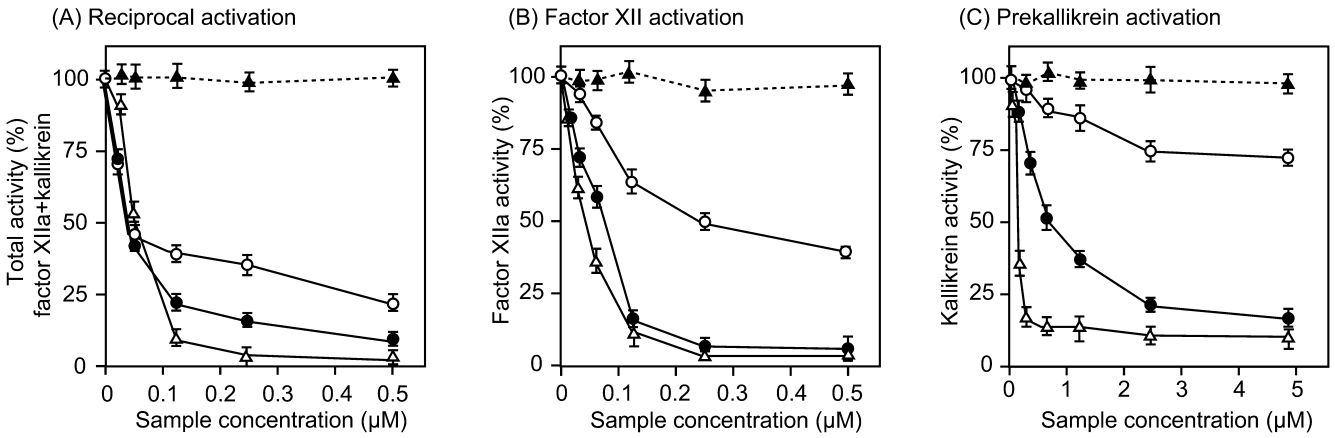

Fig. 4. **Inhibitory effects of the N-terminal and C-terminal domains on reciprocal activation of factor XII and prekallikrein.** The inhibitory effects of the N-terminal domain (open circles), C-terminal domain (solid circles), and haemaphysalin (open triangles) on activation of the kallikrein-kinin system were investigated through reconstitution assays involving factor XII/XIIa and prekallikrein/kallikrein. In all assays, a control experiment was performed using lysozyme (solid triangles). (A) Effects of the recombinant proteins on the reciprocal activation of factor XII and prekallikrein. Factor XII (final concentration, 0.2 nM) was preincubated for 10 min with samples at room temperature. The autoactivation of factor XII and following reciprocal activation were started by the addition of prekallikrein (10 nM) and DS500 (0.2 μg/ml). After 10 min incubation, S-2302 (170 μM) was added, and then the increase in absorbance at 405 nm was monitored. (B) Effects of samples on activation of factor XII catalyzed by kallikrein. Factor XII (20 nM) was preincubated with samples for 10 min at room temperature. The activation was initiated by the addition of kallikrein (0.2 nM) and DS500 (0.3 μg/ml). After 10 min incubation, the factor XIIa generated was measured using S-2302 (340 μM) with SBTI (0.6 μM), an inhibitor, to minimize kallikrein activity. (C) Effects of samples on activation of prekallikrein catalyzed by factor XIIa. Factor XIIa (50 pM) was preincubated with samples for 10 min at room temperature. Prekallikrein (10 nM) and DS500 (0.1 μg/ml) were added, and then activation of prekallikrein started. After 5 min incubation, the kallikrein activity generated was monitored after the addition of S-2302 (170 μM) with CTI (100 nM), a factor XIIa inhibitor, to minimize factor XIIa activity.

indicating that they could recognize the Zn^{2+}-induced conformational change of factor XIIa. Their interaction with factor XIIa in the range of 10 μM to 50 μM did not change, suggesting that they could recognize the conformational change of factor XIIa at a Zn^{2+} ion concentration of 10 μM (Fig. 5, C and D).

The binding region for haemaphysalin on factor XII was in the fibronectin type II domain, the cell-binding domain of factor XII. Thus, binding of each domain of haemaphysalin to the fibronectin type II domain was further examined. In the absence of Zn^{2+} ions, both domains weakly interacted with the fibronectin type II domain (Fig. 6, A and B). When the concentration of Zn^{2+} ions increased up to 10 μM, the binding response of the N-terminal domain to the fibronectin type II domain dramatically increased, but that of the C-terminal domain did not (Fig. 6, A and B). Binding of the N-terminal domain nearly reached a plateau at a Zn^{2+} ion concentration of 10 μM. When the Zn^{2+} ion concentration was increased up to 50 μM, the binding response of the C-terminal domain to the fibronectin type II domain changed (Fig. 6B). These results indicated that both domains bound to factor XII by recognizing the Zn^{2+} ion–induced conformational change of the fibronectin type II domain, and that the effect of Zn^{2+} ions on the interaction with the fibronectin type II domain differed between the N-terminal and C-terminal domains.

Interactions with High Molecular Weight Kininogen—We next investigated the interaction of each domain of haemaphysalin with HK by SPR spectrometry. In the presence of Zn^{2+} ions, both domains could bind strongly to HK (Fig. 7, A and B, and Table 2). While in the absence of Zn^{2+} ions, they weakly bound to HK (Fig. 7, A and B). However, in the absence of Zn^{2+} ions, their binding responses to HK did not change despite increasing concentrations of both domains. Thus, we considered that these interactions in the absence of Zn^{2+} ions were artificial (data not shown). Hence we concluded that both domains interacted with HK only in the presence of Zn^{2+} ions.

Domain D5, the cell-binding domain of HK, was earlier found to be the binding region of haemaphysalin. Thus, we examined the interaction of each domain of haemaphysalin with domain D5. Indeed, both domains could bind to domain D5 in the presence of Zn^{2+} ions (Fig. 8, A and B). In contrast, they barely interacted with domain

Table 1. **Kinetic constants of interaction between the N- and C-terminal domains and factor XII and factor XIIa in the absence of Zn^{2+} ions.**

Ligand	N-terminal domain			C-terminal domain		
	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)
factor XII	$3.76 \pm 0.01 \times 10^4$	$8.03 \pm 0.18 \times 10^{-3}$	$2.13 \pm 0.04 \times 10^{-7}$	N.D.	N.D.	N.D.
factor XIIa	$3.26 \pm 0.05 \times 10^5$	$5.95 \pm 0.04 \times 10^{-3}$	$1.70 \pm 0.24 \times 10^{-8}$	$2.54 \pm 0.18 \times 10^5$	$4.99 \pm 0.06 \times 10^{-3}$	$1.99 \pm 0.16 \times 10^{-8}$

Kinetic binding constants were obtained from sensorgram curves using kinetic evaluation software installed in the BIAcore 3000. The kinetic fitting model used in the analyses was a simple 1:1 Langmuir binding model.
N.D., not determined because the C-terminal domain did not interact with factor XII in the absence of Zn^{2+} ions

Table 2. **Kinetic constants of interaction between the N- and C-terminal domains and proteins of the kallikrein-kinin system.**

Ligand	N-terminal domain				C-terminal domain			
	k_{a1} (M^{-1} s^{-1})	k_{d1} (s^{-1})	k_{a2} (M^{-1} s^{-1})	k_{d2} (s^{-1})	k_{a1} (M^{-1} s^{-1})	k_{d1} (s^{-1})	k_{a2} (M^{-1} s^{-1})	k_{d2} (s^{-1})
factor XII	$2.81 \pm 0.26 \times 10^5$	$2.32 \pm 1.23 \times 10^{-2}$	$2.11 \pm 0.24 \times 10^{-2}$	$1.33 \pm 0.43 \times 10^{-5}$	$2.55 \pm 1.68 \times 10^5$	$4.77 \pm 0.55 \times 10^{-2}$	$1.96 \pm 0.78 \times 10^{-2}$	$9.03 \pm 0.20 \times 10^{-4}$
factor XIIa	$3.06 \pm 0.02 \times 10^5$	$1.79 \pm 0.05 \times 10^{-2}$	$5.39 \pm 0.37 \times 10^{-2}$	$5.93 \pm 1.37 \times 10^{-6}$	$2.11 \pm 0.01 \times 10^5$	$1.18 \pm 0.06 \times 10^{-2}$	$1.25 \pm 0.07 \times 10^{-2}$	$4.62 \pm 1.37 \times 10^{-6}$
HK	$2.00 \pm 0.99 \times 10^5$	$3.30 \pm 0.38 \times 10^{-3}$	$4.36 \pm 1.20 \times 10^{-3}$	$1.95 \pm 0.19 \times 10^{-4}$	$3.86 \pm 0.05 \times 10^5$	$1.99 \pm 0.16 \times 10^{-2}$	$1.34 \pm 0.90 \times 10^{-2}$	$1.18 \pm 0.70 \times 10^{-5}$

Kinetic binding constants were obtained from sensorgram curves using kinetic evaluation software installed in the BIAcore 3000. The kinetic fitting model used in the assay was a two state binding model.

D5 in the absence of Zn^{2+} ions. These results indicated that the N-terminal and C-terminal domains bound to HK by recognizing the Zn^{2+} ion-induced conformational change of domain D5.

We further examined the interactions of the N-terminal and C-terminal domains with the cell-binding region and the Zn^{2+} ion-binding region on domain D5, using synthetic peptides. Two peptides, HK421–466 and HK459–513, corresponded to the Zn^{2+} ion binding region and cell-binding region on domain D5, respectively (*19*, *41*). Peptide HK383–420 did not include either region. It has been reported that the free Zn^{2+} ions concentration in the plasma is considerably low (<0.15 μM), and that activation of the kallikrein-kinin system proceeds in the Zn^{2+} ion concentration range of 5 to 10 μM (*42*). Thus, the assay for the interaction between the two domains and HK421–466 was performed with a Zn^{2+} ion concentration of 10 μM, while assays for their interactions with HK383–420 and HK459–513 were performed in the absence of Zn^{2+} ions. Both domains bound to HK421–466, but not to HK383–420 (Fig 9, A and B). While the C-terminal domain could interact with HK459–513, the N-terminal domain did not. These results indicated that the C-terminal domain could bind to the cell-binding region on domain D5, but that the N-terminal domain could not.

DISCUSSION

Haemaphysalin is a unique kallikrein-kinin system inhibitor. Haemaphysalin could inhibit intrinsic coagulation despite that it did not inhibit the amidolytic activities of any intrinsic coagulation factors. In an earlier study, we suggested that haemaphysalin inhibits reciprocal activation of factor XII and prekallikrein by interfering with the association of factor XII and the prekallikrein-HK complex with a biologic activating surface, leading to inhibition of the kallikrein-kinin system activation (*36*). Thus, we suggested that haemaphysalin inhibits the intrinsic coagulation through this inhibitory mechanism. In this study, in order to elucidate this unique inhibitory mechanism in detail, the contribution of the N-terminal and C-terminal domains to inhibition of activation of the kallikrein-kinin system was investigated.

We suggest that both domains can inhibit the intrinsic coagulation without affecting the amidolytic activities of intrinsic coagulation factors, and showed that they could inhibit the kallikrein-kinin system by inhibiting all steps of reciprocal activation of factor XII and prekallikrein. Furthermore, both domains bound to the cell-binding domains, the fibronectin type II domain and domain D5, of factor XII and HK, respectively. These results indicated that both of them could inhibit activation of the kalllikrein-kinin system through the same mechanism to that for haemaphysalin, showing that they were involved in inhibition of the kallikrein-kinin system.

A reconstitution experiment showed that the C-terminal domain exhibited stronger inhibitory activities toward reciprocal activation, factor XII activation, and prekalllikrein activation than the N-terminal domain. These results indicated that the C-terminal domain mainly contributed to the inhibition of activation of the kallikrein-kinin system by haemaphysalin, while the N-

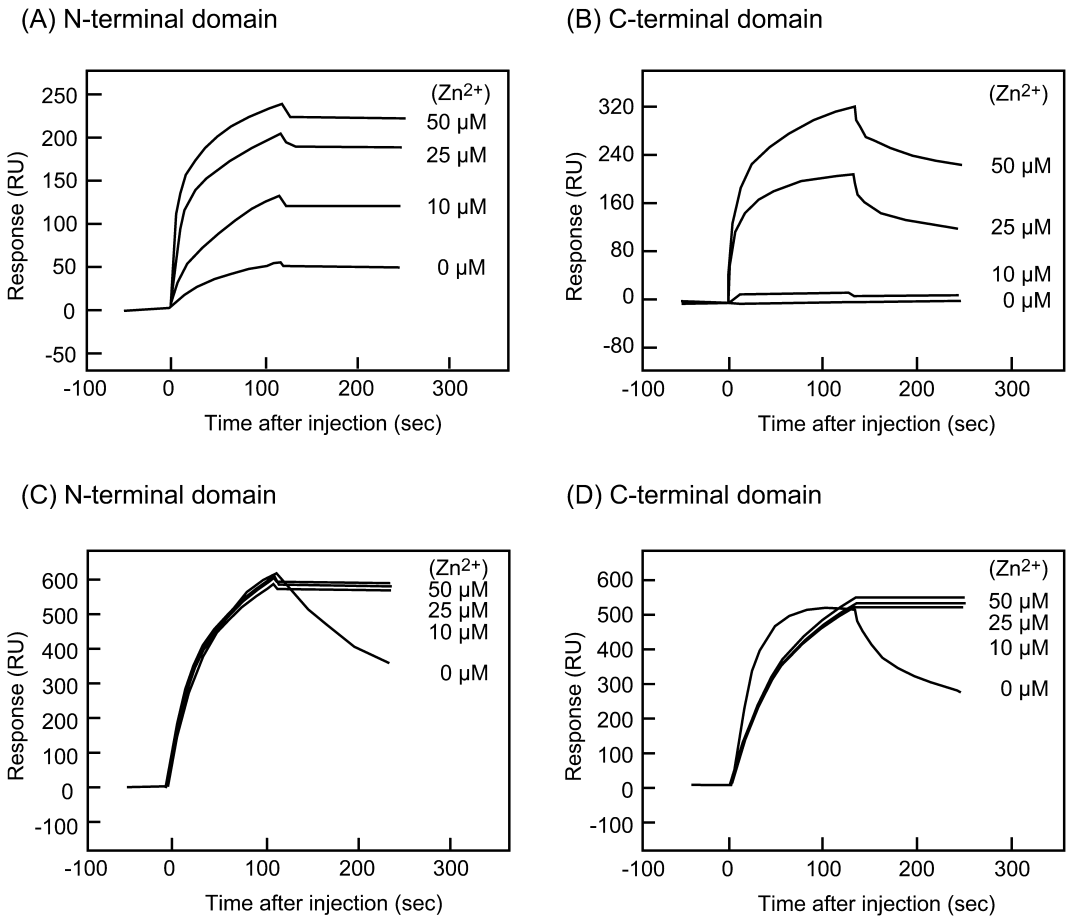

Fig. 5. **Interaction of the N-terminal and C-terminal domains with factor XII/XIIa.** Interactions of the N-terminal (A) and C-terminal (B) domains with factor XII were examined in the presence and absence of Zn^{2+} ions. The N-terminal domain and C- terminal domain were immobilized on a sensor chip at levels of 720 and 747 RU, respectively. Factor XII (300 nM) was injected onto each domain-immobilized sensorchip with various concentrations of Zn^{2+} ions (0–50 μM) at a flow rate of 20 μl/min. The association and dissociation reaction was monitored for 2 min. The surfaces of sensor chips were regenerated by injection of 1 M NaCl and 50 mM EDTA. Interactions of the N-terminal domain (C) and C-terminal domain (D) with factor XIIa were also examined. Factor XIIa (100 nM) was injected onto the surface of sensor chips under each condition at a flow rate of 20 μl/min.

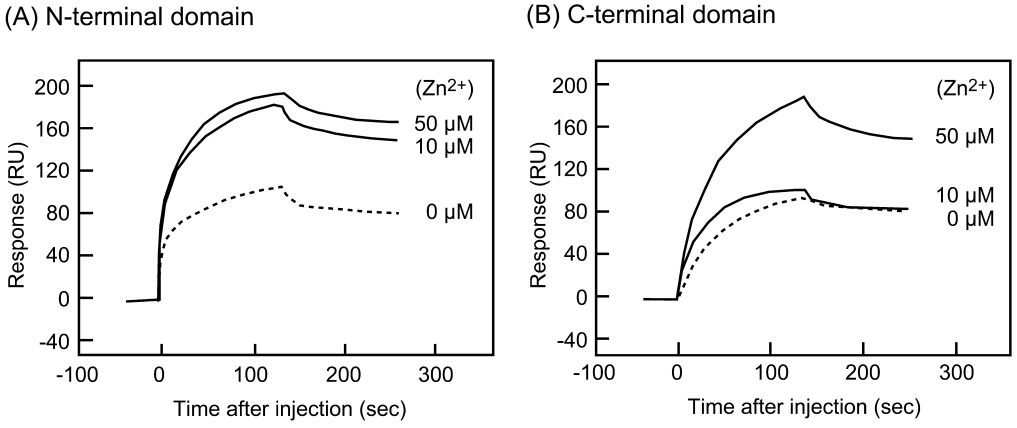

Fig. 6. **Interaction of the N-terminal and C-terminal domains with the fibronectin type II domain.** Binding of the N-terminal (A) and C-terminal (B) domains to the fibronectin type II domain was investigated. In this assay, the N-terminal and C-terminal domains were immobilized on sensor chips at levels of 1055 (A) and 1321 RU (B), respectively. Their binding was monitored in the absence and presence of Zn^{2+} ions (10 and 50 μM). The fibronectin type II domain was injected onto a sensor chip at a flow rate of 20 μl/min. The association and dissociation reaction was monitored for 2 min. Regeneration of the sensor chip surface was achieved by pulse-injections of 1 M NaCl and 50 mM EDTA. The solid and dashed lines are sensorgrams in the presence and absence of Zn^{2+} ions, respectively.

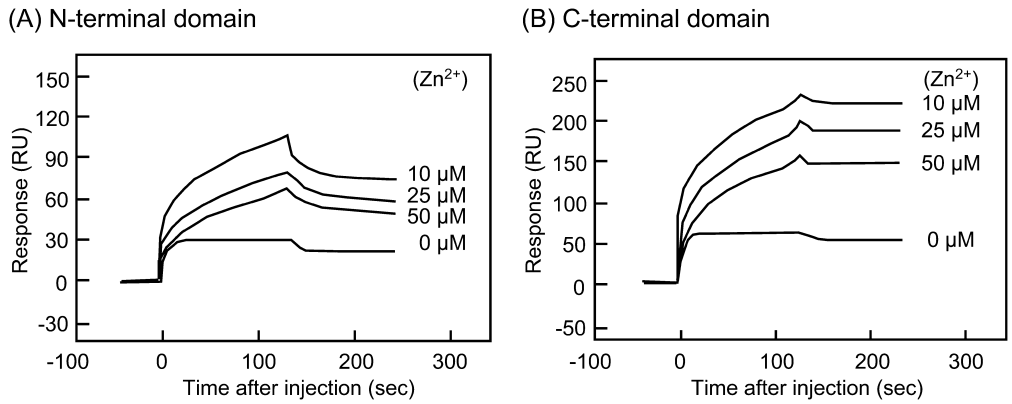

Fig. 7. **Interaction of the N-terminal and C-terminal domains with high molecular weight kininogen.** The same sensor chips as in Figure 4 were used in assays. Interactions of the N-terminal (A) and C-terminal (B) domains with HK (100 nM) were examined at a flow rate of 20 μl/min in the absence and presence of Zn^{2+} ions (10 μM). The association and dissociation reaction was monitored for 2 min. The sensor chips were regenerated by pulse-injection of 1M NaCl and 50 mM EDTA.

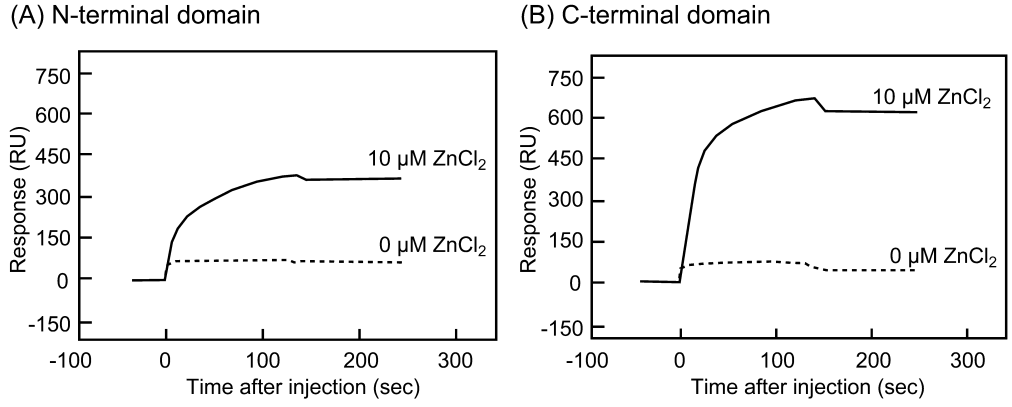

Fig. 8. **Interaction of the N-terminal and C-terminal domains with domain D5.** The some sensor chips as in Figure 4 were also used in this assay. Domain D5 of HK was injected onto the surface of sensor chips on which the N-terminal domain (A) and C-terminal domain (B) had been immobilized. The association and dissociation reaction was monitored for 2 min at a flow rate of 20 μl/min. Regeneration of sensorchips was achieved by a same procedure as described in other assays. Assays were performed in the absence and presence of Zn^{2+} ions (10 μM). The solid and dashed lines are the sensorgrams in the presence and absence of Zn^{2+} ions, respectively.

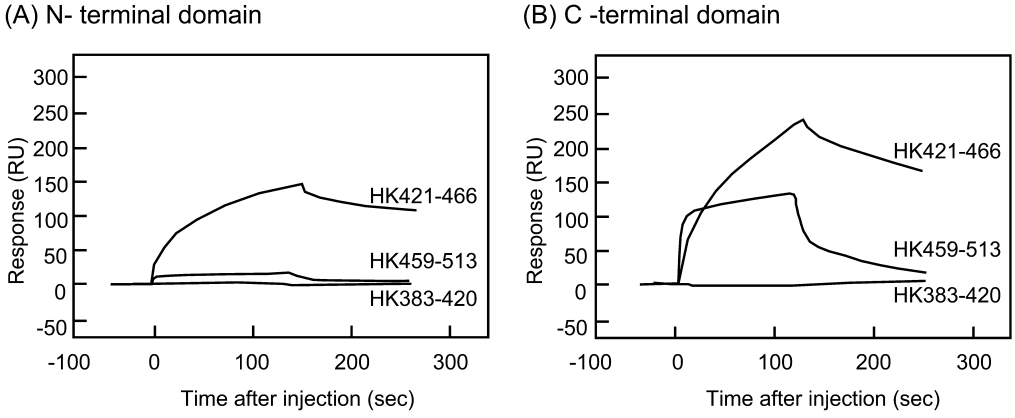

Fig. 9. **Sensorgrams of interaction of the N-terminal and C-terminal domains with peptides derived from domain D5.** The N-terminal and C-terminal domains were immobilized on sensor chips at levels of 1021 (A) and 978 RU (B), respectively. Three peptides (1 μM), HK383–420, HK421–466 and HK 459–513, were injected onto the sensor chips at a flow rate of 20 μl/min. Binding of the N-terminal and C-terminal domains to HK421–466 was examined in the presence of 10 μM Zn^{2+} ions, because this peptide includes the Zn^{2+} ion binding region of domain D5 of HK. In contrast, interactions of the two domains with the other two peptides, HK383–420 and HK459–513, were examined in the absence of Zn^{2+} ions. The association and dissociation reaction was monitored for 2 min at a flow rate of 20 μl/min. The sensor chips were regenerated by pulse injection of 1 M NaCl and 50 mM EDTA.

terminal domain played a secondary role in this inhibition, albeit that the inhibitory mechanisms are similar.

Our SPR analyses showed that the C-terminal domain bound to the cell- binding region of domain D5 of HK. In contrast, the N-terminal domain could not bind to this region of domain D5. It has been reported that this cell-binding region of domain D5 could interfere with the binding of factor XII to a biologic activating surface, suggesting that the cell-binding region on the fibronectin type II domain exhibits structural similarity with that on domain D5 despite significant differences in the overall structures between these domains (*17*). Thus, we assumed that the C-terminal domain could bind to the cell-binding region on the fibronectin type II domain as well as that on domain D5. We considered that the C-terminal domain could more effectively inhibit the association of the prekallikrein-HK complex and factor XII with a biologic activating surface by binding directly to their cell-binding regions than the N-terminal domain.

The N-terminal domain, on the other hand, only bound to the Zn^{2+} ion binding region on domain D5. A molecular modeling study suggested that the Zn^{2+} ion binding region is located structurally close to the cell-binding region on domain D5 (*43*, *44*). Thus, the N-terminal domain might bind in the vicinity of the cell-binding region on domain D5 and weakly hinder the association between HK and a biological activating surface.

Our SPR analyses showed that the effect of Zn^{2+} ion on the interaction with the fibronectin type II domain differed between the N-terminal and C-terminal domains. The fibronectin type II domain has several Zn^{2+} ion binding sites (*13*). Thus, this finding suggested that binding sites on the fibronectin type II domain differed between the N-terminal and C-terminal domains. The difference in binding sites on target molecules between the two domains of haemaphysalin would result in the difference in their inhibitory activities as to activation of the kallikrein-kinin system.

Consequently, we suggested that the N-terminal domain and the C-terminal domain could inhibit activation of the kallikrein-kinin system through a similar mechanism to that for haemaphysalin. However, the contribution of each domain to the inhibiton differed. The C-terminal domain plays a predominant role in inhibition of kallikrein-kinin system activation. The C-terminal domain likely binds to cell-binding regions on factor XII and HK. The C-terminal domain might more efficiently prevent factor XII and HK from binding to a biologic activating surface than the N-terminal domain. Thus, this domain exerted more potent inhibitory activity as to activation of the kallikrein-kinin system than the N-terminal domain. On the other hand, the N-terminal domain might bind in the proximity of the cell-binding regions on target molecules. Thus, this domain plays an additional role in inhibition. The common receptor for factor XII and HK has not been identified yet. These domains of haemaphysalin may prove useful molecular tools for identification of this putative receptor and for studies on the kallikrein-kinin system.

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