

Functional characterization of Kunitz domains in hepatocyte growth factor activator inhibitor type 2

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Abstract Hepatocyte growth factor activator inhibitor type 2 (HAI-2) was identified as a potent inhibitor of hepatocyte growth factor activator (HGF activator). The primary translation product of HAI-2 contains two Kunitz domains. To characterize their function, we introduced a point mutation into the reactive site of each Kunitz domain, and assayed the mutants for their HGF activator inhibitory activity. A point mutation in the COOH-terminal Kunitz domain did not affect the activity of HAI-2, whereas a point mutation in the NH₂-terminal Kunitz domain markedly reduced the activity. These results suggest that the NH₂-terminal Kunitz domain is mainly responsible for the HGF activator inhibitory activity of HAI-2.

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Key words: Hepatocyte growth factor; Scatter factor; Hepatocyte growth factor activator; Hepatocyte growth factor activator inhibitor; Kunitz domain

1. Introduction

Two Kunitz-type serine protease inhibitors, that efficiently suppress the proteolytic activity of hepatocyte growth factor activator (HGF activator) in vitro, were isolated from the conditioned medium of a human stomach carcinoma cell line, and designated HGF activator inhibitor type 1 and type 2 (HAI-1 and HAI-2) [1,2]. HGF activator is a serine protease that converts the inactive single-chain precursor of HGF to the active heterodimer in response to tissue injury [3–5]. The proteolytically activated HGF may be involved in regeneration of the injured tissues [6–8]. Thus, HGF activator is a specific enzyme that regulates the activity of HGF in injured tissues. Because HAIs are potent inhibitors of HGF activator, they may be involved in regulating the activity of HGF by suppressing the HGF-converting activity of HGF activator.

Recently, cDNA cloning of two Kunitz-type serine protease inhibitors was reported [9,10]. A cDNA clone was isolated from a human placenta cDNA library using the consensus sequence of Kunitz domains obtained from the expressed sequence tag data base. A protein which is encoded by the cDNA was purified from human placenta, and named placental bikunin. The purified protein exhibited potent inhibitory activity against trypsin, plasma kallikrein and plasmin [9]. Another cDNA clone was isolated by a large scale screen for differentially expressed genes in pancreatic cancer. North-

ern blot analysis using the isolated cDNA as a probe showed that the gene named *kop* was highly overexpressed in pancreatic cancer cell lines and in pancreatic cancer tissues [10]. Comparison of the cDNA sequences of HAI-2, placental bikunin and the *kop* product revealed that all three proteins are identical.

The primary translation product of HAI-2, which was deduced from the cDNA sequence, consists of 252 amino acids with a calculated molecular mass of 28 kDa including an NH₂-terminal signal peptide sequence of 27 amino acids [2]. It contains two Kunitz domains and a COOH-terminal hydrophobic region of 24 amino acids. This characteristic structure suggests that HAI-2 is first produced in a membrane associated form and then secreted from producing cells in a COOH-terminally truncated form by proteolytic processing. Because HAI-2 has two Kunitz domains, it is important to elucidate which Kunitz domain is responsible for the inhibitory activity of HAI-2 against HGF activator. We thus constructed cDNAs encoding HAI-2 proteins which have a point mutation in a putative reactive site of each Kunitz domain. The cDNAs were stably transfected into Chinese hamster ovary (CHO) cells, and expressed proteins were assayed for the inhibitory activity against the HGF-converting activity of HGF activator. A protein with a mutation in the COOH-terminal Kunitz domain (Kunitz II) had comparable activity with the wild-type protein, whereas a protein with a mutation in the NH₂-terminal Kunitz domain (Kunitz I) had much weaker activity than the wild-type protein. These results suggest that the NH₂-terminal Kunitz domain is mainly responsible for the inhibitory activity of HAI-2 against HGF activator.

2. Materials and methods

2.1. Construction of expression plasmids for HAI-2 mutant cDNAs

A cDNA encoding a secreted form of HAI-2 (sHAI-2) was constructed by polymerase chain reaction (PCR) using the full length HAI-2 cDNA as a template and two oligonucleotide primers, 5'-GAGTCGACCATGGCGCAGCTGT-3' and 5'-CCCTGCAGCTGGCGAAGCAGC-3'. The PCR product was digested with *Sa*I and *Pst*I and inserted into the *Xho*I and *Pst*I sites of an expression plasmid vector pME18S [11]. A cDNA encoding a mutant HAI-2 which had a point mutation (R48L) in Kunitz I was constructed with a Quick change site-directed mutagenesis kit (Stratagene) using pME18S-sHAI-2 as a template and two oligonucleotide primers, 5'-GTGGGCAGATGCCTGGCCTCCATGCC-3' and 5'-GGCATGGAGGCCAGGCATCTGCCCCAC-3'. A cDNA encoding a mutant HAI-2 which had a point mutation (R143L) in Kunitz II was constructed with the same kit using pME18S-sHAI-2 as a template and two oligonucleotide primers, 5'-CACTGGGCCTTGCCCTGCATCCTTCCC-3' and 5'-GGGAAGGATGCAAGGCAAGGCCAGTG-3'. A cDNA encoding a mutant HAI-2 which had point mutations (R48L and R143L) in both Kunitz domains was also constructed with this kit using pME18S-sHAI-2 (R48L) as a template and the two oligonucleotide primers used for construction of pME18S-sHAI-2 (R143L).

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2.2. Expression of plasmids for HAI-2 mutant cDNAs in CHO cells

CHO cells were cultured in a 60-mm culture dish in a 1:1 mixture of DMEM and Ham's F12 medium containing 10% fetal bovine serum (FBS). At 60–80% confluence, 5 µg of each expression plasmid and 0.5 µg of pRSVneo were cotransfected into CHO cells by the lipofectamine method [12]. After transfection, the cells were cultured in the medium containing 0.5 mg/ml of G418 and 10% FBS. G418-resistant colonies were selected and screened for expression of the mutant HAI-2 proteins by immunoblotting.

2.3. Preparation of a polyclonal antibody against bacterially expressed HAI-2

The HAI-2 cDNA was digested with *Bam*HI and *Sma*I (nucleotide residues 181–534), and inserted into the *Bam*HI and *Sma*I sites of a plasmid vector pGEX-4T-2 (Pharmacia). A fusion protein with glutathione-S-transferase was expressed in *Escherichia coli*. After sonication, cell lysate was recovered by centrifugation at 45000×g and the fusion protein was bound to glutathione-Sepharose 4B beads (Pharmacia). After the Sepharose beads had been washed three times with phosphate buffered saline (PBS) containing 0.3 M NaCl, the fusion protein was eluted with PBS containing 5 mM glutathione. Rabbits were immunized with the purified fusion protein, and antiserum (anti-HAI-2) was raised.

2.4. Immunoblotting analysis of HAI-2 mutant proteins

Each CHO cell line that stably expressed HAI-2 mutant proteins was cultured in a 150-mm culture dish in the medium containing 10% FBS. At confluence, the medium was replaced with 25 ml of serum-free medium. After 50 h, the conditioned medium was harvested and concentrated to 0.5 ml. Fifty µl of the concentrated sample was separated by SDS-PAGE (15% polyacrylamide) under reducing conditions. The proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham), and incubated with the anti-HAI-2 antiserum for 3 h. This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were visualized with the enhanced chemiluminescence immunoblotting detection system (Amersham).

2.5. Assay for HGF activator inhibitory activity of HAI-2 mutant proteins

The concentration of the mutant proteins in the conditioned medium was determined by immunoblotting analysis. Five µl of HGF activator (0.3 ng/µl) was mixed with various concentrations of the mutant proteins in 40 µl of PBS containing 0.05% CHAPS and incubated at 37°C for 30 min. Five µl of single chain HGF (1.6 µg/µl) was added to the mixture which was further incubated for 14 h. After incubation, HGF was bound to SP-Sepharose beads. After the Sepharose beads had been washed three times with PBS, HGF was eluted with 50 µl of 250 mM Tris-HCl (pH 6.8) containing 10% mercaptoethanol, 4% SDS, 20% glycerol and 0.004% bromophenol blue and separated by SDS-PAGE (12.5% polyacrylamide) under reducing conditions. The gel was stained with Coomassie Brilliant Blue, and the bands were scanned using a Flying-Spot Scanner CS-9000 (Shimadzu). The inhibitory activity was estimated by calculating the ratio of the remaining single chain form to total HGF.

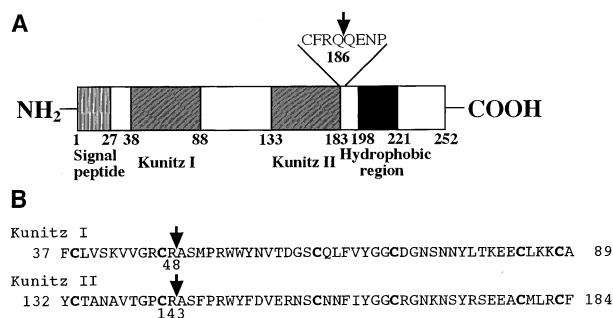


Fig. 1. A schematic of the structure of the primary translation product of HAI-2 (A) and amino acid sequences of the Kunitz domains in HAI-2 (B). In A, the truncated site used to produce a secreted form of HAI-2 is indicated by an arrow. In B, putative reactive sites are indicated by arrows. The conserved cysteine residues are shown in bold.

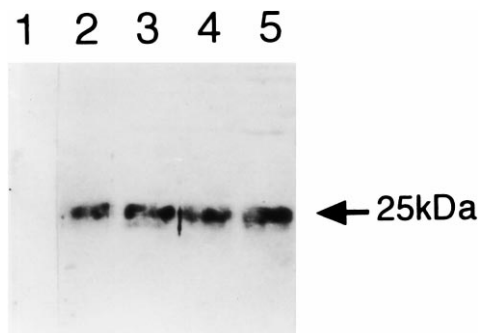


Fig. 2. Immunoblotting analysis of mutant HAI-2 proteins. Proteins in the conditioned medium of each CHO cell clone were separated by SDS-PAGE (15% polyacrylamide) and transferred to a nitrocellulose membrane. The membrane was incubated with anti-HAI-2 antiserum and developed with peroxidase-conjugated secondary antibodies. Lane 1: Parental CHO cells; lane 2: sHAI-2; lane 3: sHAI-2 (R48L); lane 4: sHAI-2 (R143L); lane 5: sHAI-2 (R48L/R143L).

3. Results

3.1. Construction and expression of HAI-2 mutant cDNAs

The primary translation product of HAI-2 has a hydrophobic sequence at the COOH-terminal region (Fig. 1A), suggesting that the secreted form of HAI-2 obtained from the conditioned medium is generated by proteolytic processing of a membrane associated form [2]. Thus, it is possible that large amounts of HAI-2 protein remain in cells in the membrane associated form. To obtain enough mutant protein in conditioned medium, we first constructed a cDNA encoding a deletion mutant that lacked the hydrophobic sequence. For construction of the deletion mutant, the cDNA sequence corresponding to the COOH-terminal end of Kunitz II was fused to the vector sequence, generating a truncated form of HAI-2 with an additional 9 amino acid sequence derived from the vector sequence (Fig. 1A). This construct was transfected into CHO cells, and a cell clone which stably expressed the secreted form of HAI-2 (sHAI-2) was obtained. Immunoblotting analysis revealed that a HAI-2 protein with a molecular mass of 25 kDa was secreted in the conditioned medium (Fig. 2, lane 2). Because the molecular mass was larger than that (19 kDa) predicted from the amino acid sequence of the truncated HAI-2, the secreted HAI-2 protein probably contained the whole amino acid sequence including Kunitz I and II. Thus, this cDNA was used to construct HAI-2 mutants with point mutations in the Kunitz domains. The larger molecular mass may be due to glycosylation of the protein, because the HAI-2 protein purified from the conditioned medium of MKN45 cells was highly glycosylated [2]. The amino acid residues in the P1 positions of the reactive sites of various Kunitz domains were assigned as essential for inhibitory activity [13,14]. Thus, each arginine residue (Arg⁴⁸ or Arg¹⁴³) in the corresponding position of each Kunitz domain in HAI-2 (Fig. 1B) was individually changed to a leucine residue. A cDNA encoding a HAI-2 mutant in which both the arginine residues were changed to leucine residues was also constructed. CHO cell lines which stably produced and secreted each mutant protein were obtained. Immunoblotting analysis showed that enough mutant protein with the same molecular mass as the wild type, 25 kDa, was obtained in the conditioned medium (Fig. 2, lane 3–5). These proteins were assayed for HGF activator inhibitory activity.

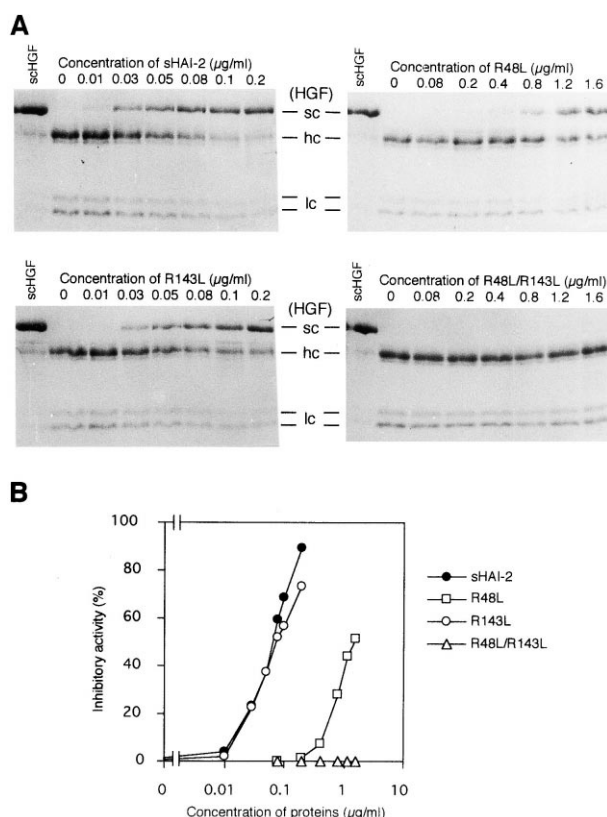


Fig. 3. Dose dependence of the inhibitory activity of mutant HAI-2 proteins against HGF activator. Various concentrations of the mutant proteins were incubated with HGF activator. After incubation at 37°C for 30 min, single chain HGF (scHGF) was added and further incubated. The reaction products were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (A). The inhibitory activity was determined as the ratio of the remaining single-chain HGF to total HGF and plotted against protein concentration (B). sc, hc and lc indicate the single chain, heavy chain and light chain of HGF, respectively.

3.2. Assay for HGF activator inhibitory activity of HAI-2 mutant proteins

Fig. 3 shows the dose dependence of the inhibitory activity of the mutant proteins. HGF activator was mixed with various concentrations of the mutant proteins and incubated for 30 min to form an enzyme-inhibitor complex. Single chain HGF was then added and further incubated. The mixture was analyzed by SDS-PAGE (Fig. 3A). The inhibitory activity of mutant proteins was determined as the ratio of the remaining single chain HGF to total HGF and plotted against protein concentrations (Fig. 3B). A mutation in Kunitz II (R143L) did not affect the inhibitory activity of HAI-2, when compared with the wild type. On the other hand, a mutation in Kunitz I (R48L) markedly reduced the activity of HAI-2. The concentration of the R143L protein for 50% inhibition was 80 ng/ml, whereas that of the R48L protein was 1.6 μg/ml. Thus, the R48L protein was 1/20 less potent than the R143L protein. No activity was observed in the protein with point mutations in both Kunitz domains (R48L/R143L).

4. Discussion

The amino acid residues in the P1 positions of the reactive sites of Kunitz domains were assigned as essential for the

inhibitory activity against serine proteases [13,14]. Both Kunitz domains in HAI-2 have arginine residues in the corresponding positions [2]. Further, the site of cleavage by HGF activator to generate the active heterodimeric form of HGF is an arginine residue [15]. Thus, these arginine residues in the Kunitz domains were expected to function as reactive sites for the inhibitory activity of HAI-2 against HGF activator. The HAI-2 protein with replacement of these arginine residues by leucine residues completely lost the inhibitory activity. The HAI-2 proteins with replacement of each arginine residue by a leucine residue retained the inhibitory activity, although the potency of activity of the protein with a mutation in Kunitz I was much lower than that with a mutation in Kunitz II. These results indicate that the arginine residue is essential for the inhibitory activity of each Kunitz domain of HAI-2 against HGF activator.

The HAI-2 mutant with the intact Kunitz I and inactive Kunitz II had potency of inhibitory activity similar to the wild type, whereas the mutant with the inactive Kunitz I and intact Kunitz II exhibited much less activity than the wild type. These results suggest that the NH₂-terminal Kunitz domain (Kunitz I) is mainly responsible for the inhibitory activity of HAI-2 against HGF activator. Delaria et al. [16] reported that placental bikunin, identical to HAI-2, inhibited trypsin, plasma kallikrein, tissue kallikrein and plasmin. They also reported that the chemically synthesized COOH-terminal Kunitz domain of placental bikunin exhibited potent inhibitory activity against these serine proteases similar to the synthesized NH₂-terminal Kunitz domain [16]. Thus, the COOH-terminal Kunitz domain (Kunitz II) in HAI-2 may be responsible for inhibiting serine proteases other than HGF activator. Hepatocyte growth factor activator inhibitor type 1 (HAI-1) obtained from the conditioned medium of MKN45 cells was also a COOH-terminally truncated form which appeared to be generated from the membrane associated form of the primary translation product [1]. The primary translation product of HAI-1 contains two Kunitz domains, and the HAI-1 protein in the conditioned medium of MKN45 cells probably contains only the NH₂-terminal Kunitz domain [1]. The identity of the amino acid sequences is 54% between the NH₂-terminal Kunitz domains in HAI-1 and HAI-2, whereas it is only 41% between the two Kunitz domains in HAI-2. We previously examined the HGF activator inhibitory activity of β-amyloid precursor protein (APP), tissue factor pathway inhibitor (TFPI) and inter-α-trypsin inhibitor (IαTI) which have Kunitz domains responsible for the inhibitory activities of these proteins against serine proteases [17–19]. The HGF activator inhibitory activities of APP and TFPI were very weak, and that of IαTI was not detected [2]. The identity of the amino acid sequences is less than 40% between each Kunitz domain in these proteins and Kunitz I in HAI-2. These findings suggest that some of the different amino acid residues contribute to distinct substrate specificity of each Kunitz domain.

IαTI is a high molecular weight glycoprotein with two tandemly repeated Kunitz domains in the light chain, which is one of three polypeptide chains linked by a glycosaminoglycan [19]. A protease inhibitor, which consists of only the COOH-terminal Kunitz domain in the light chain of IαTI [20], has been identified from mast cells [21]. This inhibitor, named trypstatin, inhibits factor Xa, trypsin, trypsin and chymase. Thus, in the case of IαTI, there is a proteolytic processing pathway to generate an inhibitor with only the

COOH-terminal Kunitz domain. The secreted form of HAI-2 which was purified from the conditioned medium of MKN45 cells as an inhibitor against HGF activator is a proteolytically processed product of the putative membrane associated form. This processing appears to occur within Kunitz II of HAI-2 [2]. Like the pathway producing trypstatin, there may be another proteolytic processing pathway to produce an inhibitor with only Kunitz II of HAI-2 which inhibits serine proteases other than HGF activator. Thus, further characterization of proteolytic processing pathways of HAI-2 is required to understand in vivo functions of each Kunitz domain in HAI-2.

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