Nucleotide sequence of a cDNA encoding a common precursor of disintegrin flavostatin and hemorrhagic factor HR2a from the venom of *Trimeresurus flavoviridis*

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Abstract The venom of *Trimeresurus flavoviridis* has three disintegrins that act as platelet aggregation inhibitors by binding to integrin αIIbβ3 on platelets through its Arg-Gly-Asp sequence. We isolated the cDNA encoding the flavostatin precursor that is one of the disintegrins in *T. flavoviridis* venom. The open reading frame consisted of four regions, a pre-peptide region, a metalloprotease region, a spacer region and a disintegrin region, indicating that the flavostatin precursor belongs to the metalloprotease/disintegrin family. Surprisingly, the deduced amino acid sequence of the metalloprotease region was completely consistent with that of hemorrhagic metalloprotease HR2a, which indicated that this metalloprotease released from the flavostatin precursor functions as a hemorrhagic factor. These observations indicated that a disintegrin and a hemorrhagic metalloprotease were synthesized as a common precursor. Thus, our results support the hypothesis that a disintegrin is synthesized as a metalloprotease/disintegrin precursor and matures by cleavage from the precursor molecule.

Key words: Disintegrin precursor; Hemorrhagic metalloprotease; Snake venom; Pro-flavostatin

1. Introduction

Viperidae snake venoms contain various components that cause dysfunction of the blood coagulation system in their prey. Disintegrins are such components and function as platelet aggregation inhibitors by binding to platelet integrin αIIbβ3 through the Arg-Gly-Asp (RGD) sequence [1]. Metalloproteases are also the major active principals of the lethal toxin of viper venom. It is known that metalloproteases in snake venoms possess specific activities as hemorrhagic factors, fibrolases and pro-thrombin and factor X activators [2]. Interestingly, all cDNAs encoding disintegrin precursors had a metalloprotease region followed by a disintegrin region through a short spacer region [3–7]. Neeper and Jacobson reported the sequence of a cDNA encoding trigramin from the venom of *Trimeresurus gramineus* (now renamed *Trimeresurus stejnegeri*), but they did not translate a large nucleotide sequence that is present upstream from the region encoding the 72 residue mature trigramin [3]. Kini and Evans [4] and Takeya et al. [5] translated all deduced amino acid sequences and determined the domain structure of the trigramin precursor. Analysis of the deduced amino acid sequences of cDNAs encoding the precursors of rhodostomin from *Calloselasma rhodostoma* venom [6] and trimucrin from *T. mucrosquamatus* venom [7] also indicated that both precursors have metalloprotease and disintegrin domains similarly to trigramin. These observations suggested that both the metalloprotease and disintegrin mature by processing of a common precursor, although these corresponding mature metalloproteases have not been isolated.

The venom of *Trimeresurus flavoviridis* contains both disintegrins and hemorrhagic factors. Four hemorrhagic factors and three disintegrins have been found in the venom of *T. flavoviridis* and their amino acid sequences have been determined. The amino acid sequences of three disintegrins, flavoridin [8], triflavin [9] and flavostatin [10], are highly homologous to one another and are completely identical from residues 1 to 46. The four hemorrhagic factors found in the venom of *T. flavoviridis* are HR1A [11], HR1B [11], HR2a [12] and HR2b [12]. Both HR1A and HR1B that are high molecular mass metalloproteases have disintegrin-like and Cys-rich domains following the metalloprotease domain [13,14]. The low molecular weight metalloproteases HR2a and HR2b that are composed of only a metalloprotease domain have high degrees of amino acid sequence identity [15,16].

In the present study, we isolated a cDNA encoding the full-length precursor of flavostatin and analyzed its nucleotide sequence. The deduced amino acid sequence of the open reading frame (ORF) of the flavostatin precursor indicated that it was composed of a pre-peptide domain, a metalloprotease domain, a spacer domain and a disintegrin domain. Surprisingly, the deduced amino acid sequence of the metalloprotease domain was identical to that of hemorrhagic factor HR2a. This is the first indication that a proportion of the low molecular weight hemorrhagic factors is expressed as a single chain precursor protein also containing disintegrin which matures by cleavage between the metalloprotease and disintegrin domains.

2. Materials and methods

2.1. Cloning of a HR2a/flavostatin precursor

Screening for cDNA encoding disintegrin was carried out by a PCR-based method for high stringency screening of a DNA library [17]. A *Trimeresurus flavoviridis* venom gland cDNA library [18] was used as the template. Approximately 8.4×10^5 clones of *Escherichia coli* in the *T. flavoviridis* venom gland cDNA library were divided into 64 wells (200 μl/well, 1.3×10^4 clones/well) in a 96 well tissue culture plate (Becton Dickinson). The pooled *E. coli*s containing positive clones as determined by PCR analysis were isolated and DNA sequences analysis was performed as described below.

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2.2. PCR analysis

Highly conserved parts of the nucleotide sequence in the disintegrin region of three disintegrins, trigramin [3], rhodostomin [6] and trimucrin [7], were used as a sense primer (11–17S: AATCCGTGCTCGATGCTGC, amino acid positions 425–430 in Fig. 2) and an antisense primer (45–51AS: TCACCCCTTGCTACTCCGGCA, amino acid positions 459–465 in Fig. 2). Each PCR mixture (20 μl final volume) contained 2 pmol of each of the above primers, 1 U of Taq polymerase (Promega), 200 μM of each dATP, dCTP, dTTP and dGTP (Takara), 2 mM Mg^{2+}, 1×PCR buffer (Promega) and 0.4 μg template. PCR was performed in a Zymoreactor (ATTO) for 30 cycles of 94°C for 0.5 min to denature the template, 60°C for 1 min for primer annealing and 72°C for 1 min for extension. After PCR, samples were kept at 4°C prior to gel analysis.

2.3. DNA sequence analysis

DNA sequencing was performed by the dideoxy chain termination method using a Taq Dye Primer Cycle Sequencing kit, Taq Dye Terminator Cycle Sequencing kit (Applied Biosystems) and a nucleotide sequencer (model 373A, Applied Biosystems).

2.4. Oligonucleotide synthesis

Oligonucleotides for PCR and DNA sequencing were prepared with a DNA synthesizer (model 381A, Applied Biosystems).

3. Results

3.1. Nucleotide sequence analysis

One cDNA clone encoding disintegrin was obtained from cDNA library of *T. flavoviridis* venom gland by screening with a PCR-based method. Fig. 1 shows the full nucleotide sequence and deduced amino acid sequence of this cDNA. This cDNA was designated pro-flavostatin because the deduced amino acid sequence of its disintegrin region was identical to that of flavostatin that is isolated from the venom of *T. flavoviridis* by Kawasaki et al. [10]. Pro-flavostatin had 81 bp of 5′-UTR, 1437 bp of ORF and 481 bp of 3′-UTR. A putative poly-A signal (AATAAA) was detected at nucleotide positions 1897–1902 of the 3′-UTR. The ORF consisted of a 54 bp signal region containing the initial ATG codon, a 516 bp pre-peptide region, a 606 bp metalloprotease region, a 54 bp spacer region and a 204 bp disintegrin region containing a stop codon located at nucleotide positions 1435–1437. The cDNA sequence of pro-flavostatin was homologous to those

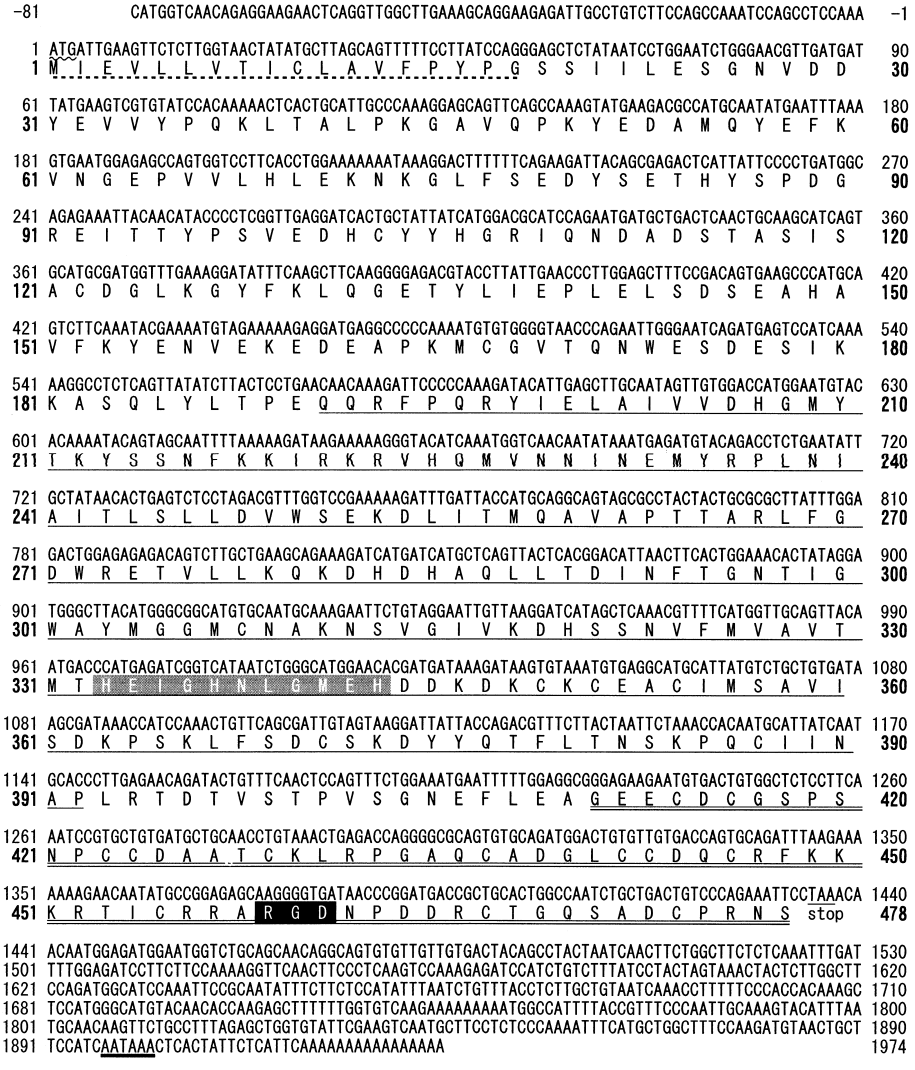

Fig. 1. cDNA and deduced amino acid sequence of pro-flavostatin, which is the precursor of the hemorrhagic factor HR2a and disintegrin flavostatin from *T. flavoviridis* venom. The nucleotide sequence is shown in the upper row, with the deduced amino acid sequence below. The initial ATG codon, putative signal peptide, hemorrhagic factor HR2a, flavostatin, Zn^{2+}-binding motif, RGD sequence and polyadenylation signal are indicated by a waved underline, dotted underline, single underline, double underline, weak reversed letters, reversed letters and bold underline, respectively.

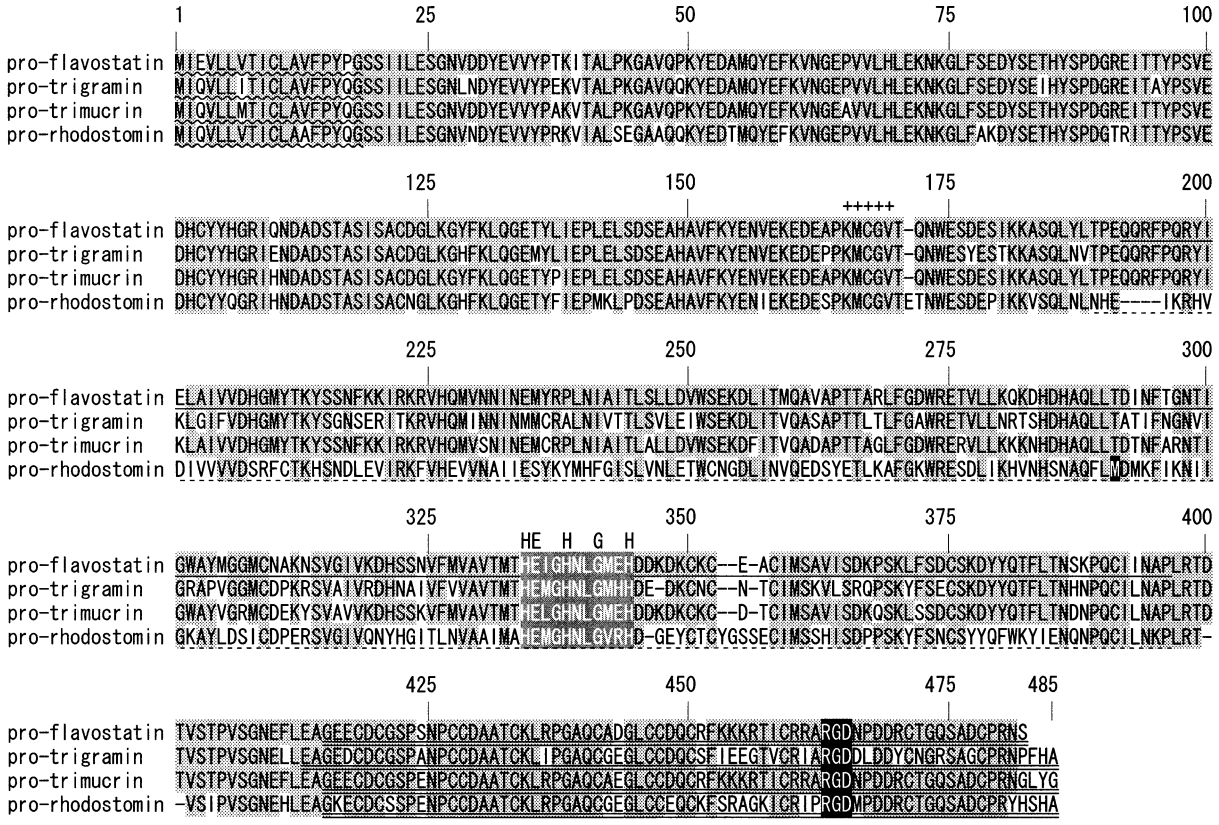

Fig. 2. Comparison of the deduced amino acid sequence of pro-flavostatin with those of other known disintegrin precursors. The single and double underlines show proteins isolated as a metalloprotease and a disintegrin from crude venom, respectively. The dotted line indicates that a highly similar protein was isolated from crude venom. Residues identical to those of pro-flavostatin are shaded. + shows the cysteine switch site [19]. Weak reversed letters indicate the Zn^{2+}-binding site and consensus residues are noted above the sequence. Reversed letters represent the Arg-Gly-Asp sequence. M, residue 291 of pro-rhodostomin, is a threonine in the amino acid sequence of rhodostoxin [20]. Pro-trigramin, *T. gramineus* [3–5]; pro-trimucrin, *T. mucrosquamatus* [7]; pro-rhodostomin, *C. rhodostoma* [6].

of the disintegrin precursors pro-trigramin, pro-rhodostomin and pro-trimucrin (data not shown).

3.2. Amino acid sequence analysis

The deduced amino acid sequence of the disintegrin region encoded by pro-flavostatin was identical to that of flavostatin [10]. Interestingly, the deduced amino acid sequence of the metalloprotease region was consistent with the amino acid sequence of the hemorrhagic factor HR2a which was analyzed by Miyata et al. [15] (Fig. 1). The deduced amino acid sequence of the spacer region, 393-LRTDTVSTPVSGNEFLEA-410, between the metalloprotease and disintegrin regions was homologous to those of the disintegrin precursors pro-trigramin [3], pro-rhodostomin [6] and pro-trimucrin [7] (Fig. 2). Furthermore, the pro-peptide region of the metalloprotease contained a predicted cysteine switch sequence KMCGV [19] located at amino acid positions 165–169 (Fig. 2).

4. Discussion

In the present study, we determined the nucleotide sequence of a cDNA encoding the flavostatin precursor. Its deduced amino acid sequence was composed of a pre-peptide domain of a metalloprotease domain, a spacer domain and a disintegrin domain of 190, 202, 18 and 69 residues, respectively. Thus, it is clear that the flavostatin precursor belongs to the metalloprotease/disintegrin family.

Interestingly, the amino acid sequence of the metalloprotease domain in the flavostatin precursor was consistent with that of HR2a, a low molecular mass hemorrhagic factor present in the venom of *T. flavoviridis* (Fig. 1). The precursors of all three sequenced disintegrins, trigramin [3–5], rhodostomin [6] and trimucrin [7], also have both metalloprotease and disintegrin domains. The only sequence analysis of a hemorrhagic factor, designated as rhodostoxin, isolated from the venom of *C. rhodostoma* confirmed the deduced amino acid sequence of the putative hemorrhagic protein encoded by the pro-rhodostomin cDNA [20]. However, amino acid sequencing revealed that residue 291 is a threonine instead of a methionine (Fig. 2). Thus, the observations that pro-flavostatin contains HR2a that functions as a hemorrhagic protein and flavostatin that acts as a platelet aggregation inhibitor disintegrin represent the first indication that a portion of the metalloprotease and disintegrin in snake venom is derived by proteolysis from a common metalloprotease/disintegrin precursor.

The metalloprotease/disintegrin precursor has three putative scissor sites between each domain, i.e. the pre-peptide, metalloprotease, spacer and disintegrin domains. Shimokawa et al. carried out an interesting experiment using Western blotting to examine processing of recombinant pro-atrolysin E, which is composed of a pre-peptide, a metalloprotease, a spacer and a disintegrin-like domain that has a Met-Val-Asp sequence instead of the RGD sequence [21]. Their results suggested

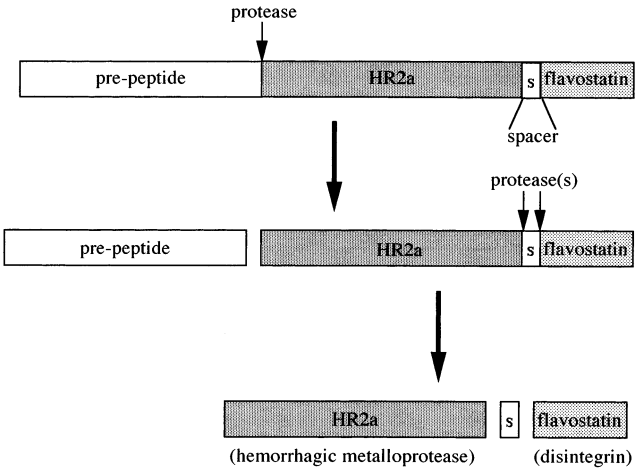

Fig. 3. Schematic model of the predicted mechanism of activation of the metalloprotease/disintegrin precursor. This is a modification of Fig. 10 of Shimokawa et al. [21]. First, the pro-flavostatin precursor seems to be cleaved between the pro-peptide and metalloprotease domains to obtain metalloprotease activity. The next cleavage site is probably between the spacer and disintegrin domain, although conclusive evidence for this has not been obtained.

that pro-atrolysin E is processed in order at the sites between the pro-peptide and metalloprotease domains, spacer and disintegrin-like domains and metalloprotease and spacer domains. Their experimental results did not allow the unequivocal determination of the final processing steps to yield atrolysin E as found in venom lacking the spacer sequence, but their study is the only one reported to date concerning metalloprotease/disintegrin precursor processing. Pro-flavostatin also belongs to the metalloprotease/disintegrin family and possesses a metalloprotease domain that matures as a low molecular weight hemorrhagic factor, HR2a, and a disintegrin domain that becomes a platelet aggregation inhibitor, flavostatin. Accordingly, the HR2a/flavostatin precursor may mature by cleavage between metalloprotease and spacer domains and spacer and disintegrin domains following proteolysis between the pro-peptide and metalloprotease domains (Fig. 3). The high molecular weight hemorrhagic factors HR1A and HR1B in the venom of *T. flavoviridis* underwent autoproteolysis, in the absence of Ca^{2+} at 37°C for 3–12 h [14]. Under these conditions, HR1A and HR1B each released a single major fragment of 32 and 34 kDa composed of spacer, disintegrin-like and Cys-rich domains. Since these metalloproteases had already lost their pre-peptide domain, the first processing site is potentially between the pro-peptide and metalloprotease domain because metalloprotease activity was observed. Next, the site between the spacer and disintegrin domains may be processed as well as pro-atrolysin E although no fragment containing a metalloprotease and a spacer domain has yet been found in the snake venom.

With the exception of the spacer domain, functions of each domain of the metalloprotease/disintegrin precursor have been proposed. It is suggested that a cysteine switch in its pre-peptide domain blocks the active site in the metalloprotease domain [19]. Its metalloprotease and disintegrin domains mainly act as a hemorrhagic factor and platelet aggregation inhibitor, respectively. However, no protein consisting of only a spacer domain has yet been isolated and its function remains unclear. Kini et al. reported that this spacer domain may not contribute significantly to the biological activity of precursor proteins because a synthetic peptide based on this spacer domain showed hardly any toxicity or biological activity [22].

In summary, we isolated and sequenced a cDNA encoding a metalloprotease/disintegrin precursor. The deduced amino acid sequence of the metalloprotease and disintegrin regions of its cDNA corresponded with those of the hemorrhagic factor HR2a [15] and the disintegrin flavostatin [9] from the venom of *T. flavoviridis*, respectively. Thus, this is the first study to clearly and directly demonstrate that the precursor of the disintegrin flavostatin is synthesized with metalloprotease hemorrhagic factor HR2a through a spacer domain and matures by proteolytic processing. The mature disintegrin and metalloprotease from the same precursor might cooperate synergistically in killing of a prey.

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