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Tachyplesin I-Induced Inhibition of Sequence-Specific Protein Binding to DNA

Atsuo YONEZAWA* and Yukio SUGIURA*

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We examine inhibition of tachyplesin I on DNA digestion with ten kinds of restriction enzymes. Tachyplesin I suppresses DNA cutting by these restriction enzymes regardless of sequence preference of the endonucleases. Competition of tachyplesin I with DNA binding of transcription factor Sp1 to GC box DNA is also investigated by using of methylation protection analysis. Tachyplesin I evidently inhibits the formation of Sp1-DNA complex. The results suggest considerably low sequence-specific DNA-binding of tachyplesin I.

KEY WORDS: Tachyplesin I / Peptide-DNA interaction / DNA binding protein / Restriction endonuclease / Competition assay

Introduction

Tachyplesin I is a small cationic peptide consisting of a rigid antiparallel β-sheet (Figure 1). The peptide exhibits strong antimicrobial activity and has broad-spectrum against Gram-positive, -negative bacteria, and fungi [1,2]. Cytotoxicity against mammalian cells and in-

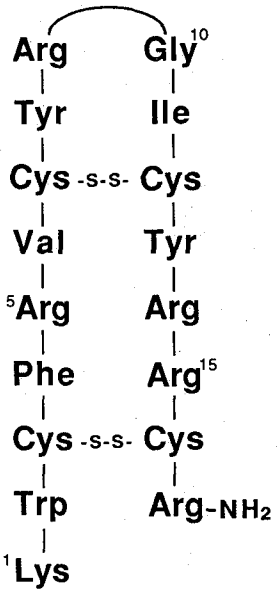

Figure 1. Chemical structure of tachyplesin I.

* 米澤敦夫、杉浦幸雄: Division of Bioorganic Chemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

hibitory effects on the proliferation of human immunodeficiency virus (HIV) are also reported [3]. Little is known about mechanism of the cytotoxicity of tachyplesin I against the various targets. Recently, we presented DNA binding property of tachyplesin I by using several footprinting techniques and suggested that DNA may be one of the biological targets of tachyplesin I [4].

In this study, we have demonstrated that tachyplesin I inhibits various sequence-specific protein binding to DNA. We investigated blocking effect of tachyplesin I on DNA cleavage by various restriction endonucleases. Methylation protection analysis was also used to study competition of tachyplesin I with Sp1 zinc finger domain [5] for GC box sequence. Sequence-specificity for DNA binding of tachyplesin I has been discussed from these experimental results.

Materials and Methods

Materials. Synthetic tachyplesin I was offered by Prof. N. Fujii (Kyoto University). The Sp1 fragment, Sp1 (167 *) was prepared and purified as described previously [5]. *Ssp*I, *Eco*RI, *Bam*HI, *Sma*I, and *Sal*I were obtained from Takara Shuzo (Kyoto, Japan). *Ban*II was purchased from Toyobo (Japan). *Eco*RV and *Rsa*I were obtained from Nippon gene (Japan). *Fok*I restriction endonuclease was a kind gift of Drs. M. Takanami and H. Sugisaki (Kyoto University). All other chemicals used were of commercial reagent grade.

Restriction Endonuclease Inhibition Assay. Linearized plasmid DNA (0.4 μg) was preincubated with tachyplesin I at 37℃ for 15 min in the respective incubation buffer (final volume, 20 μl) for each restriction enzyme in the absence of dithiothreitol. The enzyme (*Ssp*I, *Eco*RI, *Eco*RV, *Bam*HI, *Sma*I, *Sal*I, or *Fok*I) was added (10 units) to the sample and incubated at 37℃ for 60 min. The incubation with the restriction enzyme *Ban*II (2 units) or *Rsa*I (5 units) was carried out at 37℃ for 120 min or 40 min, respectively. The sample was extracted with phenol/chloroform, and subsequent extraction was performed with chloroform. Then, DNA was recovered by ethanol precipitation. The electrophoresis was performed by using a 1 % agarose containing ethidium bromide (0.5 μg/ml).

Methylation Protection Analysis. The reaction mixture (final volume, 20 μl) contained the ^{32}P end-labeled DNA fragment (pBR322 *Bst*NI-*Hae*III (105 bp)), sonicated calf thymus DNA (0.4 μg), and tachyplesin I in 20 mM Tris-HCl (pH 7.5). After preincubation at 20℃ for 15 min, Sp1 (167*) (final 2.3 μM) was added to the sample solution, and the sample was incubated for 15 min. Methylation was initiated by addition of 5 % dimethyl sulfate (DMS) (2 μl) and then the reaction mixture was incubated for 5 min. The reaction was terminated by addition of 5 μl of DMS stop solution (1.5 M acetate buffer (pH 7.0), 1 M β-mercaptoethanol, and 250 μg/ml yeast tRNA), and 75 μl of cold ethanol. The DNA was recovered by centrifugation at 15000 rpm for 10 min, and then the ethanol precipitation was carried out again. After rinsing and drying, the sample was treated with 1 M piperidine at 90℃ for 30 min. Electrophoresis was performed in a 10 % polyacrylamide / 7 M urea slab gel at 2000 V for 3 hr. After electrophoresis, an autoradiogram was made by ^{32}P end-labeled DNA in the gel. DNA sequencing was carried out by the Maxam-Gilbert method [6]. The radioactivity of each sample subjected to electrophoresis was approximately 10000 cpm.

Results and Discussion

We demonstrated DNA binding of tachyplesin I by using of several footprinting analysis [4]. In the previous study, some obvious footprints were observed. However, preferential sequences for DNA binding of the peptide were not specialized. To clarify whether the peptide has sequence-specific DNA binding or not, we examined DNA cleavage analysis with restriction endonuclease in the presence of tachyplesin I. Figures 2-4 show the effect of

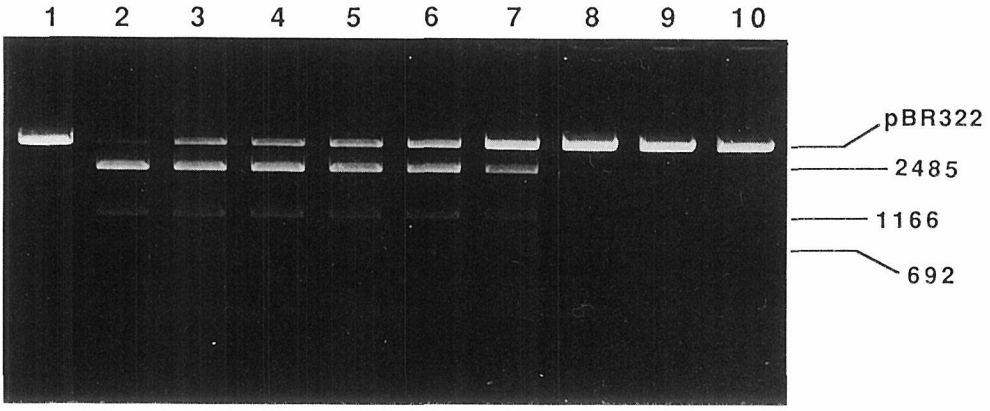

Figure 2. Effect of tachyplesin I on *Dra*I activity. *Pvu*II-linearized pBR322 fragments were digested by *Dra*I in the presence and absence of tachyplesin I. The tachyplesin I concentrations in lanes 2-10 were 0, 0.5, 1, 2, 5, 10, 25, 50, and 100 μg/ml, respectively. Lane 1 shows intact *Pvu*II-digested pBR322.

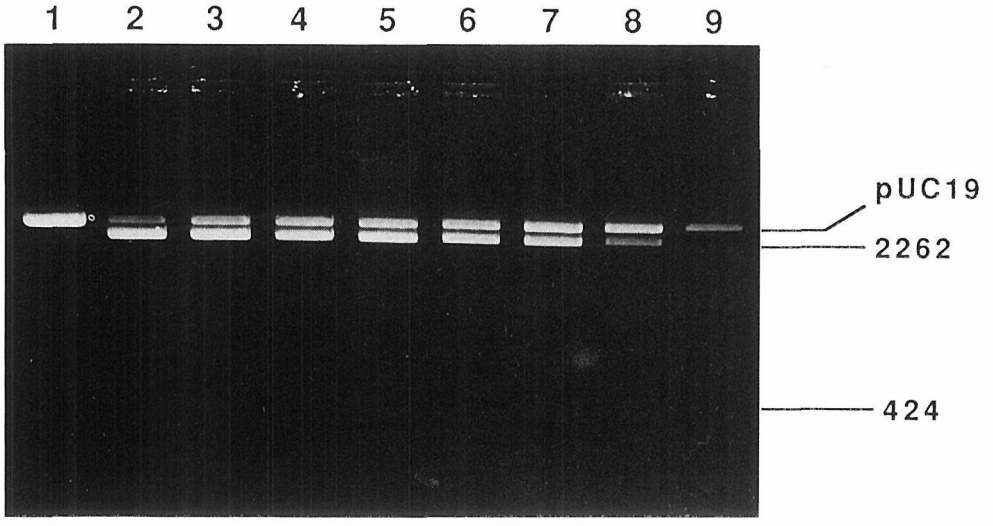

Figure 3. Effect of tachyplesin I on *Sma*I activity. *Eco*O109-linearized pUC19 fragments were digested by *Sma*I in the presence and absence of tachyplesin I. The tachyplesin I concentrations in lanes 2-9 were 0, 0.5, 1, 2, 5, 10, 25, and 50 μg/ml, respectively. Lane 1 shows intact *Eco*O109-digested pUC19.

tachyplesin I on restriction endonuclease activities. Inhibitory effect of the peptide on DNA digestion with *Dra*I which cleaves the DNA at 5'-TTTAAA-3' was apparently detected at 0.5 μg/ml (0.22 μM) (Figure 2; lane 3). Up to 10 μg/ml, tachyplesin I gradually inhibited DNA digestion by *Dra*I depended upon the peptide-concentration (lanes 2-7). Above 10 μg/ml, the inhibition dramatically proceeded, and DNA cutting with *Dra*I was completely suppressed at 50 μg/ml (lane 9). Similar inhibitory effect of tachyplesin I against digestion with *Sma*I recognizing GC-rich sites, 5'-CCCGGG-3', was also observed (Figure 3). In the case of *Sma*I, the inhibitory effect of tachyplesin I remarkably proceeded from the peptide concentration of 25 μg/ml (lanes 8 and 9). We also tested inhibition for digestion with nine kinds of restriction enzymes; *Dra*I, *Ssp*I, *Eco*RI, *Eco*RV, *Bam*HI, *Ban*II, *Sma*I, *Sal*I, and *Fok*I, by tachyplesin I at concentrations of 1, 10, 40, and 100 μg/ml. The results and the recognition sequences of these restriction enzymes studied are summarized in Table 1. Tachyplesin I evidently

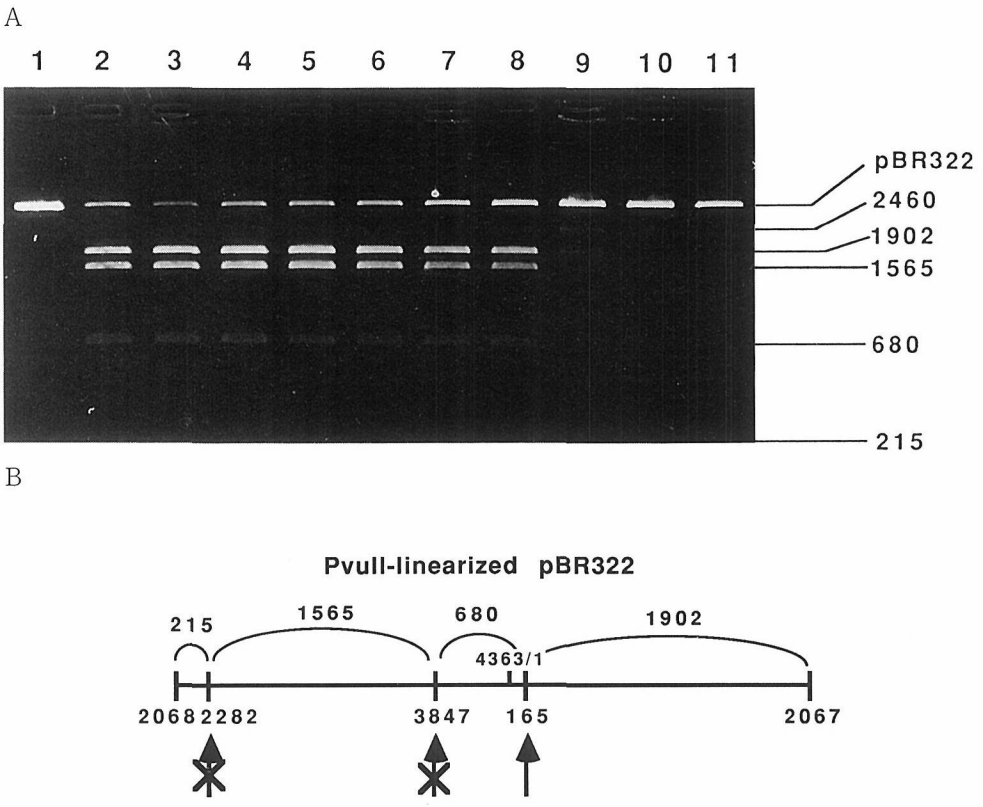

Figure 4. Effect of tachyplesin I on *Rsa*I activity. (A) *Pvu*II-linearized pBR322 fragments were digested by *Rsa*I in the presence and absence of tachyplesin I. The tachyplesin I concentrations in lanes 2-11 were 0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 μg/ml, respectively. Lane 1 shows intact *Pvu*II-digested pBR322. (B) pBR322 linearized with *Pvu*II (position 2068). The three cleavage sites for *Rsa*I are indicated by arrows. Two sites blocked by tachyplesin I are displayed by crosses superimposed on the arrows.

Table 1. *Protection of Recognition Sites of Various Restriction Endonucleases by Tachyplesin I*
After preincubation of linearized plasmid DNA (0.4 μg) with tachyplesin I at 37℃ for 15 min, enzyme was added to the sample solution and incubated. DNA was recovered by ethanol precipitation following phenol/chloroform-extract procedures. The electrophoresis was performed by using a 1 % agarose containing ethidium bromide (0.5 μg/ml).

Enzyme	Recognition Site	Inhibition of Enzyme Activity[a]			
		1[b]	10	40	100
*Dra*I	5'-TTTAAA-3'	+	+	++	++
*Ssp*I	5'-AATATT-3'	-	+	+	++
*Eco*RI	5'-GAATTC-3'	-	+	+	++
*Eco*RV	5'-GATATC-3'	+	+	+	++
*Bam*HI	5'-GGATCC-3'	±	+	+	++
*Ban*II	5'-GPuGCPyC-3'	±	+	+	++
*Sma*I[c]	5'-CCCGGG-3'	+	+	++	++
*Sal*I	5'-GTCGAC-3'	-	+	+	++
*Fok*I	5'-GGATGN_9-3'	+	+	++	++

a. Partial and complete inhibition are represented by the symbol "+" and "++".
b. Concentration of tachyplesin I (μg/ml).
c. Substrate DNA is *Eco*O109-linearized plasmid pUC19.

suppressed restriction cleavage at all of these sites. Partial or complete inhibition of restriction enzyme activities was detected at ~ 10 μg/ml or 40 ~ 100 μg/ml of tachyplesin I, respectively. It was previously demonstrated that distamycin A interferes with restriction enzyme [7,8]. This compound inhibits more effectively the activities of the enzymes recognized AT-rich DNA sites than those of the enzymes recognized GC-rich sites. In contrast, tachyplesin I inhibited evenly specific DNA cutting by many kinds of restriction enzymes. The result suggests that tachyplesin I possesses low sequence-specificity on DNA binding. Indeed, 3-azidoamsacrine and polyamine which relatively nonspecifically bind to DNA, inhibit the activities of various restriction enzymes [9,10].

Plasmid pBR322 contains three cutting sites for the restriction enzyme *Dra*I or *Rsa*I. All sites for *Dra*I were evenly blocked from enzymatic cleavage by tachyplesin I (Figure 2). Whereas, a careful titration with tachyplesin I revealed that the endonucleolytic cleavage at each recognition site of *Rsa*I was inhibited at different concentration of the peptide (Figure 4A). In the presence of 20 μg/ml of tachyplesin I, the peptide masked the two recognition sites of *Rsa*I and DNA was digested only at one site (position 165) (lane 9 in Figure 4A and illustration of Figure 4B). Similar non-equivalent inhibition by distamycin A or actinomycin D among DNA cleavage sites with restriction enzyme has also been reported [8,11]. The observation has been interpreted as differences of their flanking sequences and/or an alteration of the conformation at and around the recognition site of the enzyme. Inhibition and enhancement of DNA cleavage by bleomycin-Fe(II), which is sensitive to conformational distortion of DNA double helix [12,13], were detected in our previous result [4]. Presumably, the

non-equivalent inhibition of tachyplesin I on the digestion with *Rsa*I is caused by differences of sequences and/or conformational alterations in the vicinity of the recognition site of the enzyme by the peptide binding to DNA.

The low sequence-specificity of tachyplesin I and its binding influence on the flanking region of binding sites may cause to inhibit specific-interaction of DNA binding proteins with various target sequences. Specific binding of transcription factor Sp1 to their DNA recognition sites, GC box sequence, has been detected by methylation protection analysis [13-15]. Since tachyplesin I does not entirely inhibit or enhance methylation of the N-7 site of guanines as described in our previous paper [4], we applied the methylation protection analysis to investigate the competition of tachyplesin I with Sp1. The C-terminal 165-residues fragment of Sp1 containing three Cys_2His_2 zinc fingers, designated Sp1 (167*) [5], was used as Sp1. Figure 5 shows the competition patterns of tachyplesin I with DNA binding of Sp1 to

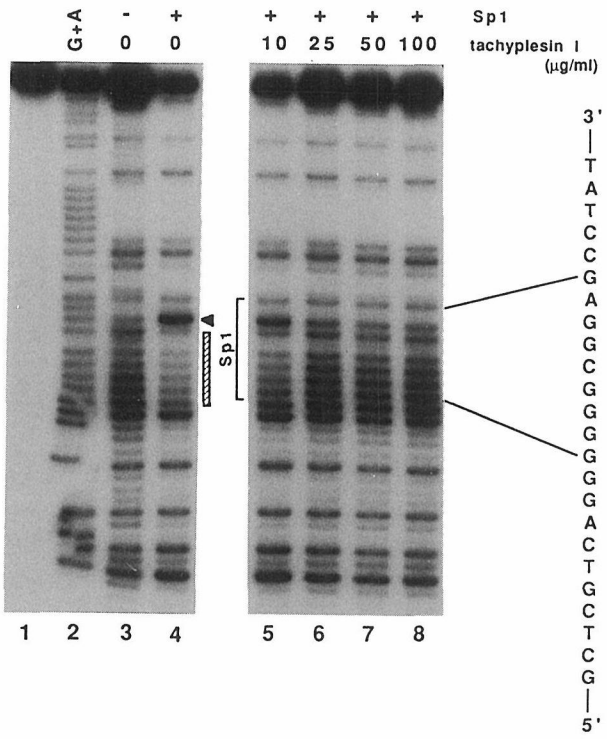

Figure 5. Competition of tachyplesin I for DNA binding of Sp1 by methylation protection analysis. Before addition of Sp1 (2.3 μM), the DNA was preincubated with the following concentration of tachyplesin I: lane 4, 0 μg/ml; lane 5, 10 μg/ml; lane 6, 25 μg/ml; lane 7, 50 μg/ml; and lane 8, 100 μg/ml. Lane 3 shows the control pattern of DMS modification. Lanes 1 and 2 exhibit intact DNA and DNA sequencing (G+A) by Maxam-Gilbert method, respectively. Arrowhead and shaded box indicate the enhanced and inhibited methylation sites by Sp1.

DNA fragment from plasmid pBR322 containing pseudo GC box. In comparison with the methylation pattern by DMS without Sp1 and tachyplesin I (lane 3), inhibition (shaded box) and enhancement (arrowhead) of methylation at guanine residues in pseudo GC box were observed in the presence of 2.3 μM Sp1 (lane 4). Preincubation of the labeled DNA fragment with tachyplesin I clearly inhibited the formation of Sp1-DNA complex depending upon the concentration of the peptide (lanes 5-8). Very recently, it was shown an instance that minor groove-binding peptide and a major groove-binding protein can simultaneously bind to a common site on DNA [16]. However, tachyplesin I which interacts in the minor groove of DNA [4] appears to influence significantly to the binding of proteins in the major groove. Our results also imply that tachyplesin I may inhibit the biological action of sequence-specific DNA-binding proteins.

Herein, we clearly demonstrate that tachyplesin I inhibits DNA digestion with various restriction enzymes and the binding of transcription factor Sp1 to GC box sequence. The results strongly suggest non-specific binding of tachyplesin I for DNA sequences.

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