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Aptamers (nucleic acid ligands) for trypsin-like serine proteases

Sang-Wan Gal†, Yong-Kee Jeong* and Satoshi Nishikawa**

Department of Microbiological Engneering, Chinji National University, Chinju 660-758, Korea
**Department of Microbiology, Dong-Eui University, Busan 614-714, Korea*
***National Institute of Bioscience and Human Technology, MITI Tsukuba Science City, Ibaraki 305, Japan*

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Abstract Subpopulations of nucleotides that bind specifically to a variety of proteins have been isolated from a population of random sequence RNA/DNA molecules. Roughly one in 10^{13} random sequence RNA/DNA molecules folds in such a way as to create a specific binding site for small ligands. Since the development of *in vitro* selection procedure, more than 50 nucleic acid ligands (aptamers) have been isolated. These molecules are very useful for the study of molecular recognition between nucleic acid and protein/organic compound. In addition to these basic studies this method gives us a dream to produce new drugs against several diseases. We focused on several aptamers which specifically binds to trypsin-like serine proteases (thrombin, human neutrophil elastase, activated protein C and NS3 protease of human hepatitis C virus) and want to introduce their structural characteristics and some functions.

Key words: aptamer, nucleic acid ligand, serine - protease

Introduction

In recent years, *in vitro* selection technique has received much attention [8] as an approach to design molecules with specific functions and in studying the structure-function relationship between nucleic acids and proteins. The general procedure of this technique is summarized in Fig. 1. The structural complexity of a combinatorial-sequence library of single-stranded DNAs/RNAs provides great potential for selection of molecules that bind to a defined target with high affinity and specificity [4,17]. Subsequent rounds of PCR amplification and selection lead to isolation of the best target-binding DNA/RNA molecules (nucleic acid ligand or aptamer) from the library. In addition to producing aptamers specific to nucleic acid binding proteins, this *in vitro* selection and amplification has thus far yielded high affinity

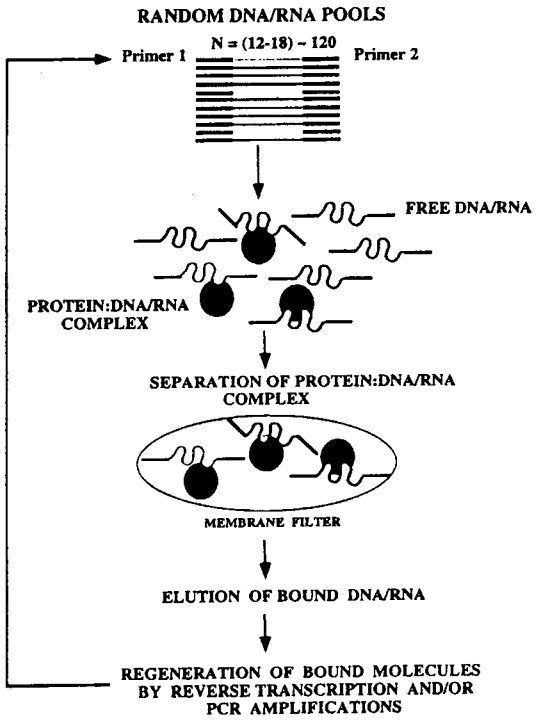

Fig. 1. Schematic flowchart of *in vitro* selection procedure.

aptamers for a number of target molecules, including non nucleic-acid-binding proteins and small molecules. Until recently several aptamers for trypsin-like serine proteases as non nucleic acid proteins have been isolated. The trypsin-like protease family are commonly found in prokaryotes, animals and viruses, surprisingly so far not found in plants. These enzymes participate in diverse physiological processses. The best known among them are digestions, fertilization, blood cloting cascade, and developmental processes. It is thought that they diverged from a common ancestral protein. Because they contain a conserved triad of catalytic residudes including Ser195, His57 and Asp102 (marked by asterisks in Fig. 2, numbering is based on chymotrypsin numbering). Another feature is that they have the same molecular con-

†Corresponding author
Phone: 82-55-751-3393, Fax: 82-55-755-2553
E-mail: sangal@cjcc.chinju.ac.kr

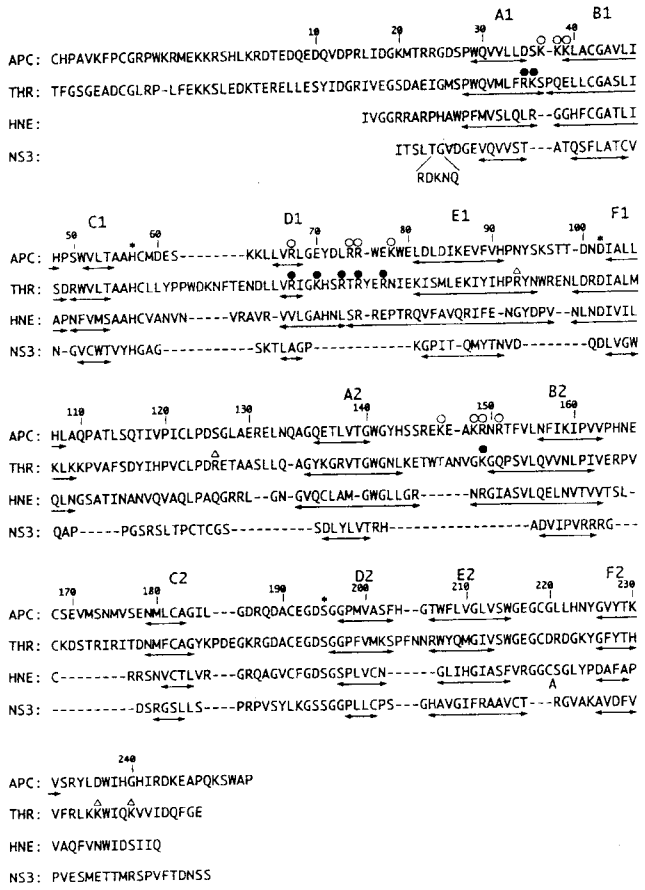

Fig. 2. Amino acid sequence alignments of several trypsin-like protease domains. Abbreviations are follows, APC; protease domain of activated protein C [5], THR: protease domain of α-thrombin [2], HNE: human neutrophil elastase [3], NS3: protease domain of human hepatitis C virus [9]. Numbering is based on chymotrypsin numbering. Underlined arrows (A1 to F1 and A2 to F2) indicate β-stranded regions. Asteriks indicate catalytic triad residues. Closed circles indicate anion binding exosite (fibrinogen and hirudin binding site) of thrombin. Triangles indicate heparin binding site. Open circles in APC indicate a similar region that corresponds to anion binding exosite of thrombin.

formation that is two domains composed of six stranded antiparallel β barrel structures (Fig. 3). Active site is located between two domains and the arrangement of the catalytic triad is well conserved among these families even amino acid sequences are quite diffferent. In this short review we describe structural chacteristics of aptamers which bind to trypsin-like proteases and inhibit their functions, and also features of binding site on protease molecules.

Thrombin aptamers

Thrombin is a central enzyme in blood coagulation and anticoagulation cascades. It converts fibrinogen to fibrin by specific proteolysis and this is essential for platelet activation. But too much thrommbin is harmful by generating thrombosis and causes many cardiovascular diseases. Accordingly antithrombotic drugs are required and ideally these

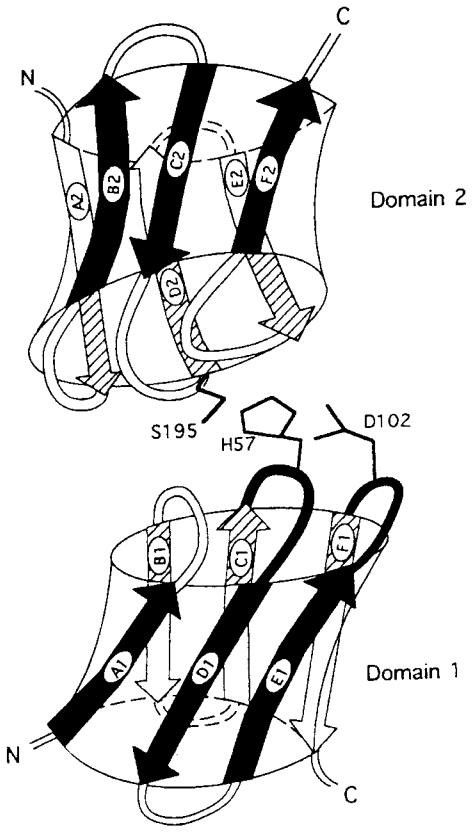

Fig. 3. Topology diagram of trypsin-like proteases. A1 to F1 and A2 to F2 show β stranded regions.

drugs should provide specific funtion without increasing risk of bleeding. In order to develop alternative efficient anticoagulants, aptamers against the human thrombin were explored [1]. Starting from a pool of DNA containing 60 nucleotides of random sequences, about 30 variants that bind to thrombin were isolated after five iterative rounds of selection and amplification. Their binding affinities to thrombin were ranging 25 ~ 200 nM. In these oligonucleotides consenses 15-mer sequence containing repeated GG sequence, $GGNTGG(N)_3GGNTGG$, was identified. The tertiary structure of this 15-mer oligonucleotide was solved by NMR [12,21] and X-ray crystallographic analysis [14]. Based on these studies it appears that the oligonucleotide folds into G-quadruplex structure which could be aligned by four guanine bases in a plane (Fig. 4A). The complax structure of the 15-mer DNA and thrombin revealed by the X-ray analysis suggests that the binding site of the DNA is anion binding exosite that is one of positively charged region of this enzyme in addition to fibrinogen binding site (marked by closed circles on thrombin sequence in Fig. 2). This result was further confirmed by chemical modification [15] and site-directed mutagenesis [18]. As expected, the selected DNA aptamer inhibited efficiently blood clotting function of thrombin by interfering with fibrinogen binding at the anion exosite with nM range. In addition to the DNA apta-

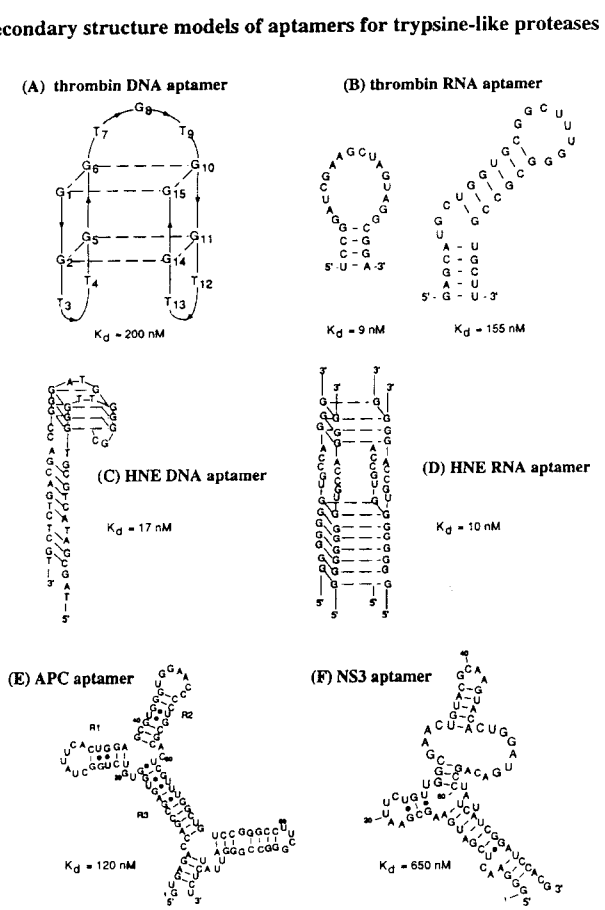

Fig. 4. Sequences of isolated aptamers and Kd values. (A) Thrombin DNA aptamer. (B) Thrombin RNA aptamers. (C) HNE DNA aptamer. (D) HNE RNA aptamer. (E) APC RNA aptamer. (F) NS3 RNA aptamer.

mer, two kinds of RNA aptamers for thrombin were isolated [6]. They have completely different sequences from DNA aptamers and exhibited stem-loop structures (Fig. 4B). Competition assay with heparin and hirudin indicated these aptamers bind to the heparin binding site this is another posistive charged region in thrombin molecule (marked by triangles in Fig. 2). These RNA aptamers did not inhibit cleavage of a small tripeptide substrate by thrombin, suggesting that they do not bind in the catalytic site of the enzyme. The structural difference of aptamers between DNA and RNA is thought to be based on the selection conditions. For the selection of DNA aptamers potassium ion (5 mM) was used in the buffer and it might enable the formation of quadruplex structure. Another major feature for binding site of the aptamer is fundamentally positive charged region.

Human neutrophil elastase aptamers

Human neutrophil elastase (HNE) is a strong serine protease and has been implicated in a number of inflammation disease. Inhibition of HNE is necessary to avoid degradation of normal tissues *in vivo*. In view of this the development of a new HNE inhibitors that have high affinity, specificity to the HNE and less toxicity *in vivo* are desirable to become an effective anti-inflammatory compounds. A DNA aptamer for HNE was isolated from DNA pool that contained a core of 40-nt randomized region [10]. Minimal motif that is sufficient to bind to the HNE found to be G rich in sequence. Structural analysis by H-NMR data suggested that the DNA folds into a G-quartet structure with duplex ends. (Fig. 4C). In spite of the high affinity binding to HNE, this molecule could not inhibit the protease activity of HNE. Moreover the DNA aptamer could efficiently bind to HNE even though the catalytic pocket was occupied by an irreversible N-methoxysuccinyl- tetrapeptide. Thus the aptamer binding site on HNE is different from the active site or substrate binding site. It is interesting to note that even though the selected ligand does not inhibit HNE activity, when conjugated the aptamer with a weak competitive inhibitor, the inhibitory property was improved several folds compared to the original inhibitor alone. To obtain nuclease resistant RNA aptamers for HNE, modified NTPs (2'-NH_2-UTP and 2'-NH_2-CTP) were used instead of natural UTP and CTP during *in vitro* transcription of RNA. This method is useful to obtain nuclease resistance RNA molecules because exogenous RNAs are usually quickly degraded *in vivo*. The selected RNA ligand had G-rich sequences and suggested inter-molecular G-quadruplex structure (Fig. 4D) based on the nuclease mapping studies [11]. As with selected DNA ligands, the RNA ligand also could not inhibit the protease activity of HNE. In both cases buffers used for selections contained potassium ions. The binding sites of HNE aptamers on HNE are not known. When we speculate from the results of thrombin aptamers, positive charged regions are one good candidate for binding sites. Actually HNE has 19 arginine and no lysine residues, and they all locate on the surface of the molecule except one as horseshoe-like manner around the active site. This may be the origin that HNE aptamers shows very strong binding constants.

Activated protein C aptamer

Activated protein C (APC) plays an important role along with thrombin as a regulator in blood clotting cascade [16]. APC functions to maintain blood fluidity as an anticoagulant by limited proteolysis of procoagulant cofactors. Substrate specificity of APC toward synthetic small peptide is similar to that of thrombin but macromolecular specificities are quite different. We succeeded to isolate an aptamer which binds to and inhibits APC from randomized 120-nt pool after seven rounds of selection and amplification [7]. The selected aptamer, APC-167 did not bind to thrombin or factor Xa and thus demonstrated precise specificity to APC. A deletion variant of APC-99 (Fig. 4E) showed the same binding ability and also efficient inhibitory function against APC with tetra-peptide substrate. The inhibition constant

(Ki) of APC-99 was 140 nM. Although we used a buffer containing potassium ions for *in vitro* selection, the proposed secondary structure of APC-99 does not seem to form G-quadruplex structure and it seems to consist of three-stems structure (R1, R2 and R3). This secondary structure was supported from the results by nuclease partial digestion and boundary analysis from both 5'- and 3'-ends. As another structural feature, every stem (R1, R2 and R3) has a tandem repeat of G · U wobble base-pairs and substitution to GC base-pairs completely diminished the binding activity suggesting at least one of these G · U wobble base-pairs is necessary for binding to APC. Phosphate modification interference analysis suggested that loops in R1 and R2 interact with APC. The kinetic analysis of cleavage reactions in the presence of APC aptamer made it clear that APC aptamer was non-competitive inhibitor. Accordingly, the binding site of APC aptamer is not the catalytic pocket and interferes the binding of the small substrate.

Recent X-ray ananlysis of APC indicates that APC has a cluster of positive charges on the surface (marked by open circles in Fig. 2) that is similar to the positively charged anion binding exosite of thrombin [13]. Furthermore this region has more basic residues than the anion exosite and is a probable binding site of APC aptamer.

HCV NS3 aptamer

NS3 protease of HCV is essential for processing of nascent transcribed viral precursor polyprotein and that means NS3 is essential for proliferation of HCV. This NS3 protein is a multifunctional protein, N-terminal domain has protease function and C-terminal domain has helicase function. Recently tertiary structure of protease domain of NS3 was solved [9] and clarified that this has trypsin-like two β barrels and a catalytic triad of His-Asp-Ser. However, NS3 protein exhibits several unusual features as a trypsin-like protease: C-terminal domain of NS3 has helicase activity, a peptide (NS4A; located immediately downstream of NS3 on the polyprotein) is necessary or helpful for the cleavage reaction. From the sequence alignment of Fig. 2, it is clear that only NS3 sequence is quite different from the others. At this moment there is no natural inhibitors for this NS3 and NS3 aptamers may become a good seed for anti-HCV drugs.

In order to screen whole random sequenced molecules, we started from a RNA pool that had a random sequence core of 12 to 18 nucleotides (containing 10^{11} molecules). Aptamers that bind specifically to NS3 protein were selected after 10 rounds of selection and amplification. A single NS3 aptamer (Fig. 4F) was found predominantly in the selected pool [5]. This aptamer could inhibit moderately NS3 proteolytic activity using synthetic peptide substrate in *vitro*. By chemical modification analysis we restricted in RNA structure, we are now trying to get more divergent molecules starting from 120-nt random RNA pool. Furthermore in order to isolate clearly specific aptamer to protease domain of NS3, we expressed only protease domain in *E. coli* [2].

Future prospects

Although this strategy is called as "irrational drug design", occationally one can achieve desired properties to the aptamers. However, using current *in vitro* selection strategy one cannot select an aptamer that binds to a predetermined site on a protein. This could be either due to inability to select aptamers that bind to the specific site on the protein or interact more weakly at the specific site. In order to select aptamers for the predetermined site of the protein, the use of monoclonal antibodies, inhibitors and substrate could be helpful during selections, where randomized nucleic acids pool and protein could be released by substrate, inhibitor or monoclonal antibodies. We are combining these approaches while screening aptamers for several viral target proteins.

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