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An RNA Aptamer That Discriminates Bovine Factor IX from Human Factor IX

Subash C.B. Gopinath[1], Dhakshnamoorthy Balasundaresan[1], Joe Akitomi[2] and Hiroshi Mizuno[2,*]

[1]Functional Nucleic Acids Group, Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566; and [2]VALWAY Technology Center, NEC Soft Co. Ltd., Shinkiba 1-18-7, Tokyo 136-8627

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An RNA aptamer has been selected by SELEX against bovine factor IX using an RNA pool containing 74-nucleotides randomized region. Selected RNA aptamer (Clone 5) could discriminate bovine factor IX effectively from human factor IX. Interestingly, the nucleotide regions 73–78 and 80–83 of the selected aptamer were determined to be important for bovine factor IX–binding using phosphate interference. Based on phosphate interference and binding studies the minimal motif for aptamer with discriminating ability is found with the nucleotide regions from 65 to106. The discriminating ability of this mini aptamer is calculated as more than 1,000 fold. The equilibrium dissociation constant (K_d) for the above complex was 10 nM as determined by surface plasmon resonance. Based on the available structural informations, probable binding site of aptamer on the target was predicted.

Key words: aptamer, bovine factor IX, discrimination, human factor IX, RNA, SELEX.

RNA aptamers are nucleic acid ligands that can be selected from random RNA pool molecules by iterative rounds of selection process involving separation and amplification of molecules have high affinity to the target. Aptamers are addressed as an important diagnostic and sensor tool alternate to antibodies (*1*, *2*) and currently using as a probe on the chip based detection (*3*, *4*). Due to its sensitivity, an interaction of aptamers and proteins with metal is studied in single-molecule level and used for the development of nanotechnology (*5*). Moreover, aptamers have also been considered to be a drug in human as a milestone for emerging drug development (*6*). In addition to that RNA aptamer was used to understand its role in the human blood coagulations systems (*7*, *8*).

In the past aptamers are shown to have high affinity to their targets with the dissociation constant in subpicomolar level as the mark to prove as comparable as and even better than antibodies (*1*). Using the advantage of sensitivity, the aptamer can be generated in the laboratory for discriminating purposes as a single probe. High discrimination was achieved by anti-theophylline aptamer that discriminates against caffeine by over 10,000 fold, eventhough the caffeine molecule differs from theophylline by only the presence of a methyl group at the N7 position (*9*). An anti–L-arginine RNA aptamer is also proved for high discrimination ability, which can discriminate L-arginine with 12,000 fold from D-arginine (*10*). Another example of selective discrimination by an RNA aptamer selected against is the cofactor Nicotinamide, the selected aptamer has the discriminating ability between the oxidised and reduced forms of Nicotinamide (*11*). Similarly, in the past, aptamers have been shown as a single probe to discriminate efficiently between closely related proteins (*12*), between peptide enantiomers (*13*), and a phosphorylated protein can be discriminated efficiently over its nonphosphorylated form (*14*). Aptamer can also discriminate the closely related viral sub-types (*15*, *16*). Developing these kinds of specific probes that distinguishes closely related species will have great impact in developing specific diagnostic reagents. Previously, an RNA aptamer which bind to bovine thrombin was selected by the process of SELEX and the selected aptamer can discriminate bovine thrombin from human thrombin (*17*, *18*). In the present investigation we have shown an RNA aptamer that dintinguishes bovine factor IX from human IX as a new member for discriminating aptamers.

EXPERIMENTAL PROCEDURES

Proteins and Generation of RNA—Bovine factors IX and IXa, Human factors IX and IXa were purchased from American Diagnostica, Inc. (Stamford, CT, USA). SELEX libraries are designed to contain a central domain of a randomized sequence flanked by 5′ and 3′ regions of invariable sequences. The ssDNA library *TCTAATACGACTCACTATAGG*AGCTCAGCCTTCACTGC–N74—GGCACCACGGTCGGATCCAC, with 74-nucleotide contiguous random sequences, was synthesized and flanked by defined sequences, including the T7 promoter (in italics). The 5′ and 3′ defined sequences were 5′-*TCTAATACGACTCACTATAGG*AGCTCAGCCTTCACTGC-3′ and 5′-GTGGATCCGACCGTGGTGCC-3′, respectively. Polymerase chain reaction (PCR) was performed with the DNA random library (1×10^{14} molecules), along with 0.25 μM each of the 5′- and 3′-primers. To preserve the abundance of the original library, the PCR was limited to eight cycles, to avoid amplifying a skewed population of the random DNA library. The DNA library was converted into the

*To whom correspondence should be addressed. Phone: +81-29-861-6097, Fax. +81-29-861-6095, E-mail: mizuno-hiroshi@aist.go.jp

RNA library by *in vitro* transcription using a T7 Ampliscribe kit (Epicentre Technologies) and was used for selection.

SELEX Procedure—Selection was performed using 15 μg ($\sim 10^{14}$ RNA molecules) of the original RNA library in binding buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) with a 4:1 molar ratio of RNA to protein. During the selection cycles, the bovine factor IX concentration was reduced in the later cycles to select high affinity RNA. The RNA was briefly heated to 95°C and cooled to room temperature, to form stable structures before the selection steps. In the selection cycles, tRNA (total tRNA from *E. coli*, Boehringer-Mannheim) was used as a nonspecific competitor. After the RNA was incubated with protein at room temperature for 10 min, the protein-RNA complexes were filtered through a pre-wetted nitrocellulose acetate filter (HAWP filter, 0.45 μm, 13.0 mm, Millipore) fitted in a "Pop-top" filter holder (Nucleopore) and washed with 1 ml of binding buffer. The RNAs that were retained on the filter were eluted and recovered as described previously (*15*, *16*). The recovered RNAs were reverse-transcribed in 20 μl of a reaction mixture, containing 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 6 mM $MgCl_2$, 0.4 mM dNTPs, 2.5 μM primer (24.N30) and 5 U of AMV reverse transcriptase (Seikagaku). Nucleotides and enzymes were added after a denaturation and annealing step (2 min at 90°C followed by an incubation at room temperature for 10 min). Reverse transcription was carried out for 1 h at 42°C. The resulting cDNA was amplified by PCR and used as the template to obtain RNA for the next round of selection. For amplification by polymerase chain reaction (PCR), a 20 μl aliquot of the mixture after reverse transcription (cDNA reaction mixture) was diluted in 80 μl of a mixture for PCR [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 5 U of Taq DNA polymerase (Takara), and 0.4 μM of each primer]. The reaction mixture was cycled at 94°C for 70 s, at 55°C for 50 s, and at 72°C for 70 s for as many cycles as needed to produce the product band with the correct size. The PCR product was precipitated in ethanol and used for transcription. Transcription *in vitro* was performed at 37°C for 3 h with an Ampliscribe T7 kit (Epicentre Technologies). After a treatment with DNase I, the reaction mixture was fractionated on an 8% denaturing polyacrylamide gel. The RNA was extracted from the gel, quantitated and used for the next cycle of selection and amplification.

We manipulated each selection cycle to ensure the specificity and high affinity of the binders to the bovine factor IX, including modifications of the ratio of RNA:protein and the buffer volumes. To remove the filter binders we used the Micro-titer plate (Xenobind) selection in the fourth selection cycle, we initially coated the well with 5 μg of bovine factor IX per milliliter of binding buffer and blocked remaining sites with BSA (3% of stock solution). The wells were then washed and used in the selections. In the selection, the RNA pool from the 3rd cycle was denatured at 90°C for 2 min and allowed to cool at room temperature for 10 min, to facilitate the equilibrium of different conformers. Then the pool RNA (1 μM) and the tRNAs (80 μM) were mixed in 100 μl of binding buffer, loaded twice into the BSA coated well, and incubated for 10 min at room temperature (25°C). The unbound RNAs were collected and loaded into the well coated with bovine factor IX. The reaction mixture was incubated further for 10 min. The unbound molecules were discarded, and the well was washed five times with 300 μl of binding buffer. The bound RNAs were recovered using a boiling 7 M urea solution, precipitated by ethanol and regenerated using RT, PCR and *in vitro* transcription.

Analysis of Aptamers and Binding Assay—To obtain the individual aptamers, the amplified PCR product from cycle 5 was directly ligated into the pCRII vector (Invitrogen), according to the manufacturer's protocol. DNA was isolated from individual clones by the alkaline-lysis method, and was sequenced with a Dye Terminator Sequencing kit [Applied Biosystems Inc. (ABI)] on a DNA sequencer (Model 373A, ABI). The secondary structures of the aptamers were predicted by the recently developed BayesFold program (version 1.01) (*19*), because this program is recommend for alignments of 3–50 closely related sequences, such as the sequence families frequently found in SELEX. To evaluate the binding activities of the RNA pools from different selection cycles, as well as the individual aptamers, internally labeled RNA was prepared using 0.5 mCi/ml [α-^{32}P]ATP, and binding studies were performed using a filter binding assay similar to that reported previously (*20*). The binding and *in vitro* transcription conditions were similar to those used for the selection, except for the molar ratio of RNA to protein (20 nM of RNA and 200 nM of factor IX). The filters were washed with 1 ml of binding buffer and air dried, and the radioactivity was quantitated with an image analyzer (BAS2000, Fuji Film). To ensure that the binding was specific, we added a 10-fold molar excess of tRNA as a nonspecific competitor in the binding reaction.

Surface Plasmon Resonance (SPR) Analysis—Kinetic measurements were conducted using BIAcore 2000 equipment with a streptravidin coated sensor-chip, from BIAcore, Sweden. The association and dissociation kinetics of the selected aptamer was carried out against bovine factor IX and also studied the binding ability with human and bovine factors. To determine the affinity constants of the selected aptamers, we prepared the aptamers with 24-mer poly(A) nucleotides at the 3′-end, which could anneal to the complementary biotinylated oligo (dT) [5′-Biotin-$(T)_{24}$-3′]. To prepare this RNA extended at the 3′ end, we used two primers: a forward primer similar to that used in the selection, and a 3′ end primer [5′-$(T)_{24}$-GTGGATCCGACC-GTGGTGCC-3′]. The double-stranded DNA template was generated by PCR and transcribed *in vitro*, as mentioned above. Similarly, to prepare the complementary sequences of the selected aptamer as the negative control, using the same primers it was generated on the DNA templates; 5′-GGAGCTCAGCCTTCACTGCGATGCGCCCGCAAAT-GCATTGCCGAATACCCCTCGACTCGCGAACTAGC-3′ and 5′-GTGGATCCGACCGTGGTGCCGAGCAAATGACC-GAAGCGTGATGGTGCTAGTTCGCGAGTCGAG-3′. PCR reaction was performed by annealing these 2 templates first with 4 cylces and further PCR cycles were carried out with primers as described above. Initially, the biotinylated oligo $(dT)_{24}$ was attached to the streptavidin (SA chip, BIAcore, Sweden) by dissolving the oligo in binding buffer (5 μM final concentration) and injecting it for 12–24 s. (RU 1,000) at a flow rate of 5 μl/min. The excess or unbound biotinylated oligo was washed with binding buffer, at a flow rate of 20 μl/min for 10 min. To analyze the binding kinetics

of the aptamer, 20 μl of aptamer was injected (100 nM final concentration) at a flow rate of 2 μl/min for 10 min, which resulted in an increase of about 1,200 RU upon aptamer binding to the complementary biotinylated oligo. Various concentrations of bovine factor IX (50–250 nM) were injected into the flow cell at a flow rate of 20 μl/min for 2 min, and the binding kinetics were evaluated. The sensor chip was then washed with a buffer solution, followed by 10 mM NaOH, before the next injection. The data were fit with a local fit of the kinetic simultaneous K_a/K_d model, assuming Langmuir (1:1) binding. For the discrimination study on biacore, the 33-mer stable RNA-aptamer, previously selected against factor IXa (*7*), was also enzymatically synthesized by *in vitro* transcription, using T7 RNA polymerase on a synthetic DNA template as described earlier (*8*).

Mapping of Phosphates Interference in Binding of Aptamer and the Target—In order to map the binding sites of the bovine factor IX within the selected aptamer, a phosphate modification assay was performed. *In vitro* transcribed RNA was labeled with pCp and T4 RNA ligase, electrophoresed on an 8% polyacrylamide/7 M urea gel, and eluted from the gel. The labeled RNA was allowed to fold into a tertiary structure by heating at 95°C for 2 min and slow cooling to room temperature. The 3′-end–labeled RNA (1,100 kcpm) was dissolved in buffer (20 mM HEPES, pH 8.0, 1 mM EDTA and 2.5 μg tRNA) and was mixed with 5 μl of saturated *N*-nitroso-*N*-ethyl-urea in ethanol. After modification at 90°C for 2 min, the reaction was stopped by the addition of 15 μg carrier tRNA, and the sample was recovered by ethanol precipitation. To allow the formation of the aptamer–bovine factor IX complex, 800 kcpm of treated sample were denatured in 25 μl of selection binding buffer at 92°C for 2 min, and allowed to cool at room temperature for 10 min. The complex was separated by passage through a nitrocellulose filter, and was partially hydrolyzed in a solution (200 μl) of 100 mM Tris/HCl pH 9.0 at 50°C for 5 min. The RNA cleavage products were recovered by ethanol precipitation and loaded on an 10% polyacrylamide/7 M urea gel. Aliquots of the alkaline hydrolysate of the aptamer and the sample digested with RNase T1 were co-electrophoresed for band identification. The gel was dried and exposed to X-ray film for autoradiography.

Preparation of Minimal Motif Aptamer—We prepared three minimal RNAs (mini-1, mini-2 and mini-3) for the selected aptamer to study its minimal motifs required to bind with bovine factor IX. To prepare mini-1 aptamer we used two primers (5′-AGTAATACGACTCACTATAGGGATCGTGGTAGTGCGAAGCC-3′ and 5′-GGACCGTGGTGCCCTCGTTTACT-3′) to amplify the nucleotides regions between 65 and 106 from full length aptamer and transcribed as above. For mini-2 we prepared two DNA templates (5′-AGTAATACGACTCACTATAGGGTAGTGCGAAGCCAGTAAA-3′ and 5′-GGTGCCCTCGTTTACTGGCTTCGCACTA-3′) and annealed. Using this annealed template, the double stranded DNA template for *in vitro* transcription was prepared using common T7 promoter primer and the second template of mini-2 aptamer. For the preparation of mini-3 the template was created with two oligos (5′-AGTAATACGACTCACTATAGGGATCGTGGTAGTGCGAAGCC-3′ and 5′-GGACCGTGGTGCCCTCTGGCTTCGCACTACCAC-3′) by annealing. From the annealed template the double stranded DNA template for RNA preparation was created with PCR using common T7 promoter primer the second template of mini-3 aptamer. To dertermine the dissociation constant on Biacore the poly(A) tailed RNA was prepared from the mini-1RNA by using the primers 5′-AGTAATACGACTCACTATAGGGATCGTGGTAGTGCGAAGCC-3′ and 5′-$(T)_{24}$-GGACCGTGGTGCCCTCGTTTACT-3′ with RT followed by PCR reactions.

RESULTS AND DISCUSSION

Selection of Anti–Bovine Factor IX Aptamers—Previously several RNA-aptamers are selected against wide variety of targets and are considered to be a single probe that can discriminate the closely related molecules (*9*, *10*, *12–16*). Aptamers are proved with very high specificity and affinity to targets, which are comparable to the affinities achieved by antibodies for antigens (*9*, *10*). In this view, the present investigation is carried out to select the RNA-aptamer against bovine factor IX. Previously an efficient RNA-aptamer was reported for human coagulation factor IXa with the dissociation constant in pico-molar order (*7*). However this aptamer could not distinguish coagulation factor IX from bovine (Fig. 1). To get the aptamer that can discriminate human and bovine coagulation factor IX, we selected an anti–bovine factor IX RNA aptamer with the starting RNA repertoire with randomized region over a 74-nucleotide. For each selection, the RNA pool and bovine factor IX were mixed and allowed to incubate for about 10 min, and then the bound oligonucleotide (Bovine factor IX–RNA complexes) are separated from the unbound oligonucleotides, either by filtration or xenoplate. Bound RNAs were recovered with hot 7 M urea and subsequently amplified by reverse transcription followed by PCR, and then the PCR products were transcribed *in vitro*. After five rounds of selection and amplification, about 54% of the RNA pool was bound to bovine IX. No significant retention of the selected RNA pool occurred on the filter in the absence of bovine factor IX, indicating that non-specific binders to filters were not co-isolated during the course of selection. After the fifth cycle of amplification and selection, PCR-derived molecules from the RNA pool were sub-cloned and sequenced. From the sequenced clones 6 kinds of repeating sequences represent 8% each from the total population were elected (Fig. 2a). All the selected 6 different sequences (clones A, E, 1, 5, 6 and 12) are checked for their binding with both bovine and human factor IX. All the clones have ability to bind with bovine factor IX, in addition except clones 1 and 5 all other clones are also able to bind human factor IX to some extend (Fig. 2b). Among the clones 1 and 5, clone 5 has higher discriminating ability between bovine and human factor IX without introduction of the counter selection against human factor IX during the selection process, and this clone was used for further studies. In Fig. 3a the selected high discriminating aptamer (clone 5) has shown with high affinity to the target with concentration dependent manner. The secondary structure of the selected aptamer was predicted with BayesFold version 1.01 (Fig. 3b) (*19*).

Determination of Equilibrium Dissociation Constants (K_d)—To determine the binding kinetics of the aptamer–bovine factor IX complex, we performed surface plasmon

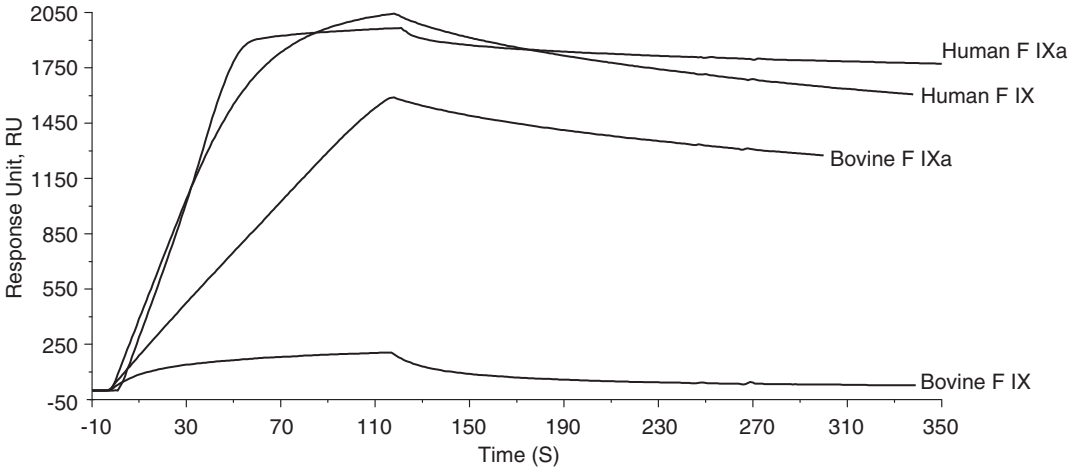

Fig. 1. **SPR-analysis for the discriminating ability of the previously reported aptamer against human factor IXa (Rusconi *et al.*, 2002).** Proteins are injected with 100 nM final concentration.

(a)

A- 5-'GGGAGCUCAGCCUUCACUGC GCGGCGGGGUAGAGCGCGAUAGGAGGAGAUGGGAAAGCGCCUCUCAAGCCGAGUGUCUGGUUAAACUCCAGGAU GGCACCACGGUCGGAUCCAC-3'

E- 5' - GGGAGCUCAGCCUUCACUGC CGAUCCGUGGCGAUCAGAUCGGCCAGGGGACACGCCAAAAGGAGGAGAUGGGAAUGAGCGCGCGACCCUGCCU GGCACCACGGUCGGAUCCAC-3'

1- 5'- GGGAGCUCAGCCUUCACUGC GUAGCGGGAGUAGGGUGAGUAGACGCGUUGAAGGAUGGUCGAGAUGUCCAUCGAAUGCGUCCUACGCGAUGCUG GGCACCACGGUCGGAUCCAC-3'

5- 5' - GGGAGCUCAGCCUUCACUGC CUACGCGGGCGUUUACGUAACGGCUUAUGGGGAGCUGAGCGCUUGAUCGUGGUAGUGCGAAGCCAGUAAACGAG GGCACCACGGUCGGAUCCAC-3'

6- 5' - GGGAGCUCAGCCUUCACUGC UCGAGGGGAAGUGGGGCCGCUGAAGUGAGCAGGCAGUCAACCGCCUCAGAUGCUCCCUUCUCUACACGCUUGGA GGCACCACGGUCGGAUCCAC-3'

12- 5'-GGGAGCUCAGCCUUCACUGC AGCCGGCAGCCGAGCAAAUAAAGAUGCUUAAGGCUACCUUCACUGUGGUGCCAGUGCGAAGACGGUAUACAGUC GGCACCACGGUCGGAUCCAC-3'

(b)

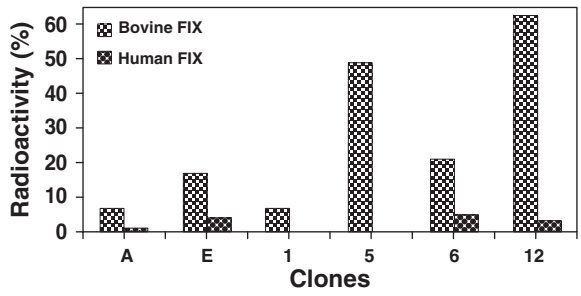

Fig. 2. **(a) Oligonucleotide sequence of selected aptamers against the bovine factor IX.** Underlined letters indicate the randomized region of the RNA. **(b) Filter binding assay to analyze the binding abilities of selected RNA aptamers with bovine and human factor IX.** Binding reactions were performed with 200 nM of protein.

resonance (SPR) studies. The kinetics studies determined by SPR revealed that the aptamer binds to the bovine factor IX with association (k_{on}) and dissociation constants (k_{off}) of 1.9×10^5 (M^{-1} s^{-1}) and 1×10^{-5} (s^{-1}), respectively. The equilibrium dissociation constant (K_d) for the above complex was 10 nM (Fig. 3c). To evaluate the specificity of the aptamer sequence, we prepared a sequence complementary to that of the selected aptamer. This complementary sequence did not bind to the bovine factor IX, suggesting the importance of the aptamer sequence.

Evaluation of Dicriminating Ability—We checked the discriminating ability of the selected aptamer (clone 5) initially by filter binding assay (Fig. 2b) and also evaluated this discriminating ability by SPR. After attaching the selected aptamer with poly-A tail, it was attached to the biotin-dT on SA-chip. To check the aptamer and factors binding, bovine and human factors IX and IXa are injected independently at 200 nM concentration through the aptamer bound flow-cell. Bovine factor IX, a 416-residue (415-residue in human factor IX) single chain molecule is activated to factor IXa by proteolytic cleavage. A cleavage removes segment Ala 147–Arg 181 (Ala 146–Arg 180 in human factor IX) to generate the active form of factor IXa. The N-terminal and C-terminal chains are disulfide linked. Thus, the difference between factor IX and IXa is only in the presence or absence of the activation peptide region. So that it is interesting to evaluate whether the aptamer can bind to both forms of factors. The obtained

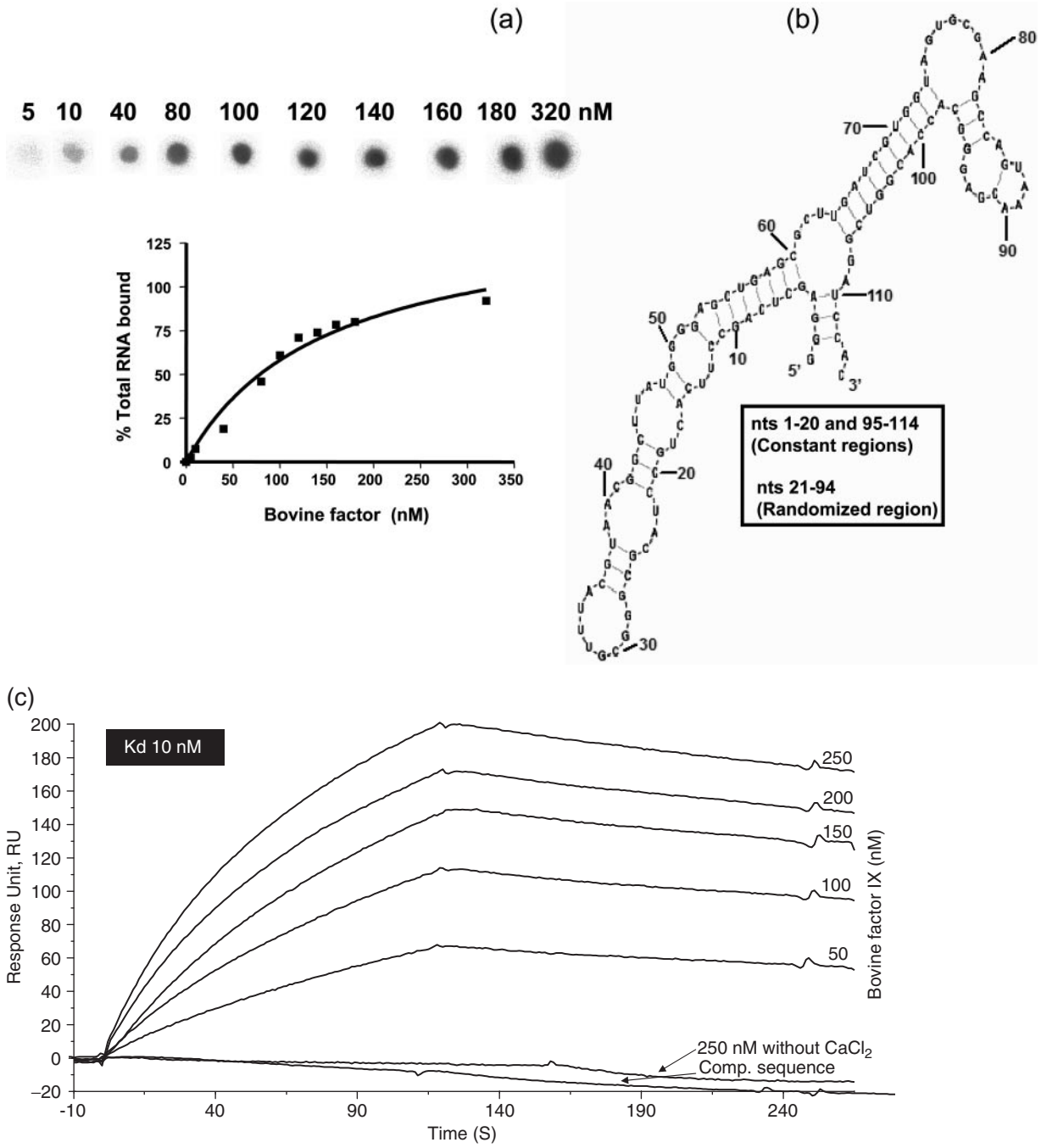

Fig. 3. **(a) Concentration dependent manner of aptamer binding with the target.** Binding reactions were performed with 10 nM of RNA against protein concentrations of 5 to 320 nM. **(b) Predicted secondary structure of the selected aptamer (BayesFold version 1.01). (c) SPR-analysis for the binding kinetics of the aptamer–bovine factor IX complex.** The apatmer was injected at a flow rate of 2 μl/min for 10 min (20 μl total volume; 100 nM final concentration). Sensogram runs with bovine factor IX injected into the flow cell, at a flow rate of 20 μl/min for 2 min. With 250 mM final concentration of bovine factor IX, the binding was also checked in the absence of calcium ions. Complementary sequences of the selected aptamer are failed to bind with 250 nM of bovine factor IX.

sensogram clearly indicates that the selected aptamer is able to discriminate between human and bovine factors (Fig. 4). But the aptamer can bind to both bovine factors IX and IXa. As discussed above, previously reported high affinity aptamer against human factor IXa (7) failed to discriminate between human and bovine factors, as it could bind to all the factors used (Fig. 1). This failure in discrimination of this aptamer might be due to the binding site of the aptamer on the target protein is in the homology regions between human and bovine factor IX. The sequence homology between human and bovine factor IX is about 85%.

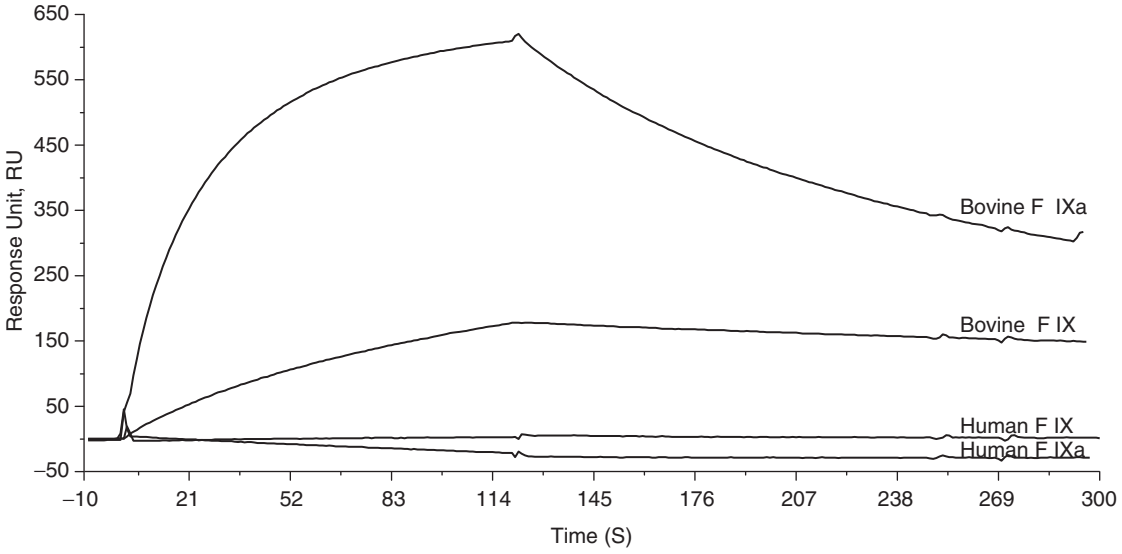

Fig. 4. **SPR-analysis for the discriminating ability of the selected aptamer.** Proteins are injected with 200 nM final concentration.

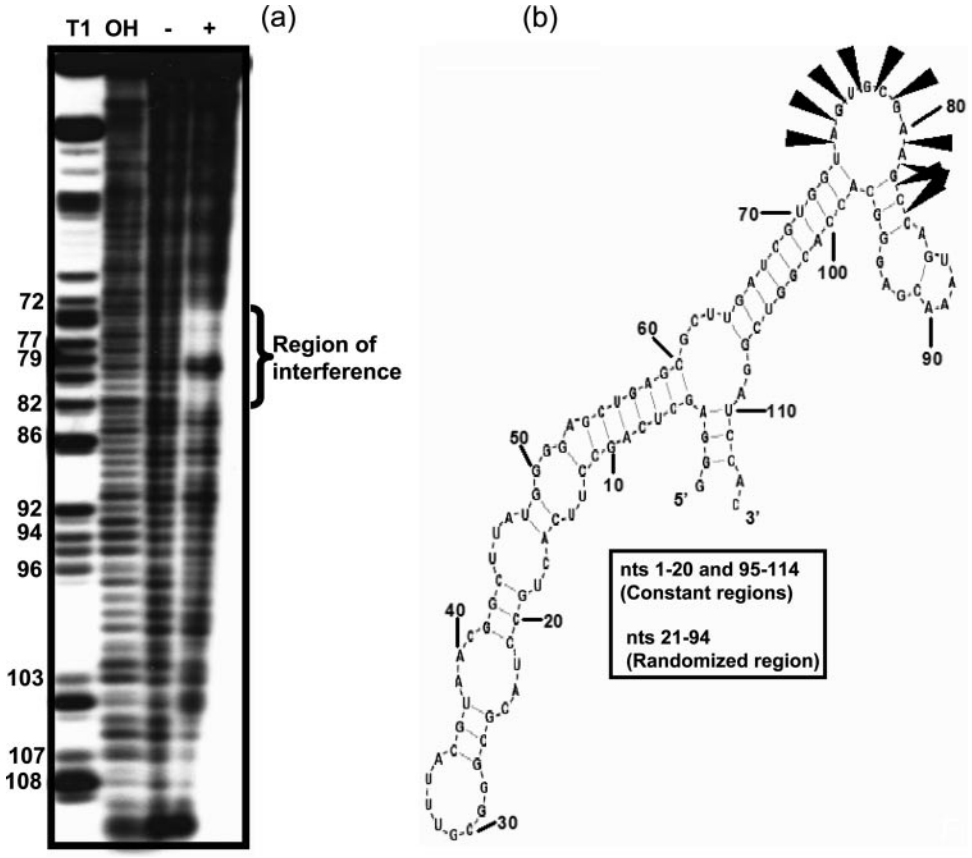

Fig. 5. **(a) Ethylnitrosourea mapping of interfering phosphates of the selected aptamer and bovine factor IX binding.** The labeled ethylnitrosourea modified aptamer-protein complex was trapped on the nitrocellulose filter. After washing the complex with binding buffer, the filter was treated with a mild alkaline solution. The RNA was recovered and loaded on the gel. The control RNA marker was prepared by partial nuclease digestion with RNase T1 (lane T1) and alkaline digestion (lane OH) Lane –: ENU RNA in the absence of bovine factor IX; Lane +: ENU RNA in the presence of bovine factor IX. **(b) Phosphate interfering regions are indicated (arrowheads) on the secondary structure.**

Phosphates Interference in Binding Complex and Minimal Motif Required—To identify the important phosphates of the aptamer (clone-5) for the complex formation, we used ENU-modifications and interference analysis methods. Initially, the selected aptamer was labeled at the 3′ end and modified under denaturing conditions (92°C for 2 min) with ethylnitrosourea. The ethylnitrosourea modified RNA was hydrolyzed by a mild alkaline treatment. The alkylated RNA was allowed to bind to bovine factor IX, and the complexed RNAs were separated from the free RNA by filtration and cleaved at the modified sites. The RNAs eluted from the nitrocellulose filters were loaded on an 10% polyacrylamide gel to separate the cleaved products. In this process, the molecules that were modified at the phosphates that are necessary for binding to bovine factor IX were lost, and these important phosphate regions could be visualized as a footprint on the sequencing gel. Comparisons of the band intensities of the samples of complexed and free RNA revealed the sites that are important for bovine factor IX binding. Specifically, the

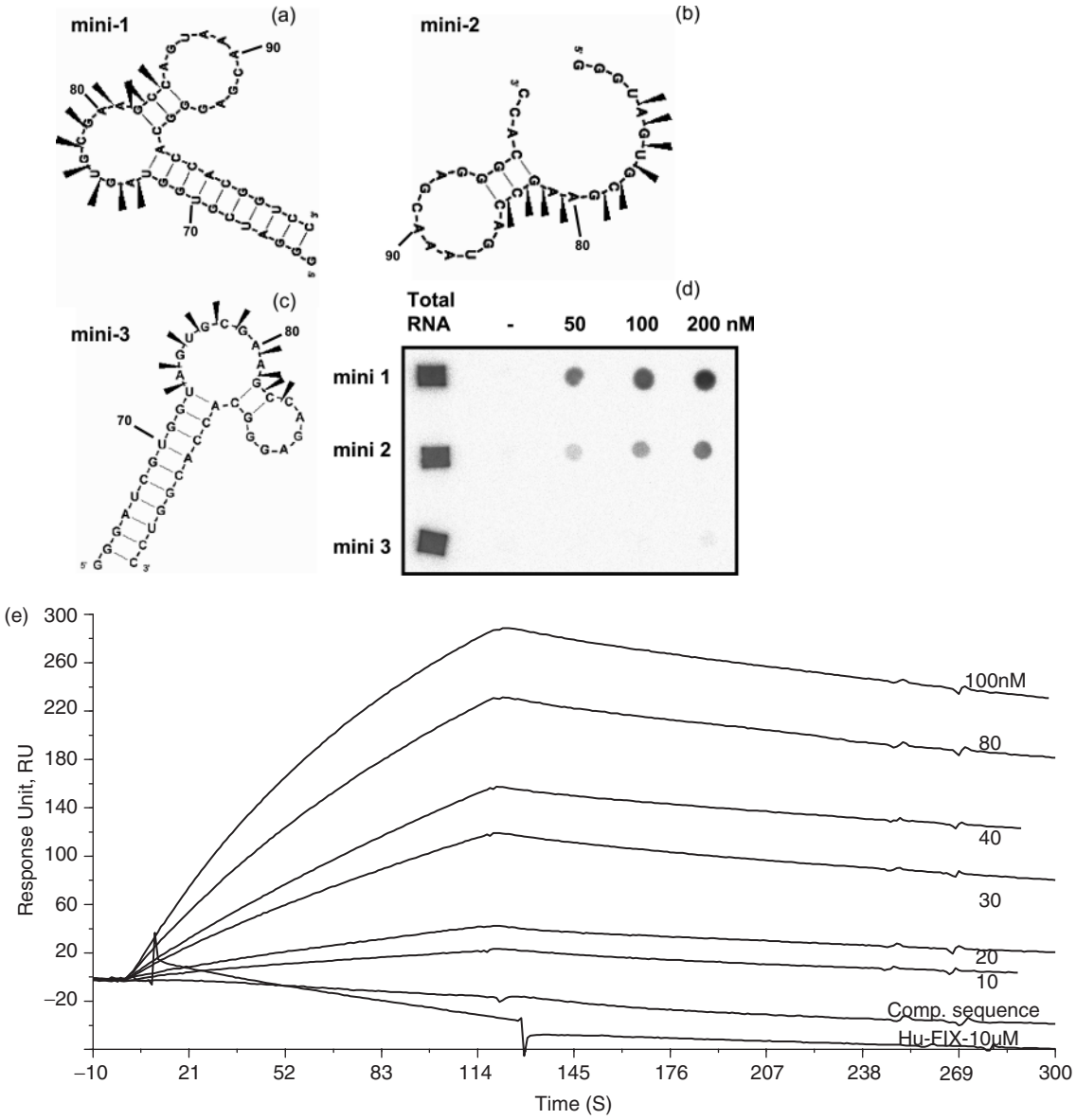

Fig. 6. **Predicted secondary structures of minimal RNA aptamers (two G residues at 5′-end and one C residue at the 3′-end were added for efficient transcription and stability of the stem, respectively).** Phosphate interfering regions are indicated (arrowheads) on each secondary structure. (a) mini-1 aptamer, (b) mini-2 aptamer, (c) mini-3 aptamer, and (d) filter binding assay to analyze the binding abilities of these minimal RNA aptamers against bovine factor IX. Binding reactions were performed with 50 to 200 nM of protein. (e) SPR-analysis for the binding kinetics of the mini-1 aptamer–bovine factor IX complex. The apatmer was injected at a flow rate of 2 μl/min for 10 min (20 μl total volume; 100 nM final concentration). To determine the discriminating ability of mini-1 aptamer with human factor IX, the protein was injected with 10 μM final concentration. Complementary sequence binding is also checked with 250 nM of bovine factor IX.

phosphates at positions U73, A74, G75, U76, G77, C78, A80, A81, G82, and C83 were found to interfere strongly with the bovine factor IX, suggesting their importance for interactions with bovine factor IX (Fig. 5, a and b). Although the base sequences were optimized during the selection, the phosphates in the random regions may also play an important role in efficient binding with bovine factor IX.

Based on the phosphate interference mapping we prepared three mini RNAs (mini-1, mini-2 and mini-3) to find the minimal RNA motif required to bind with bovine factor IX. For this study, the RNAs are prepared with the nucleotide regions are 65 to 106 (mini-1), 71 to 100 (mini-2) and 65 to 106 with the deletion of nts from 87 to 92 (mini-3). All three mini RNAs were checked for their binding using filter binding assay. Mini-1 RNA has similar affinity as determined with full length to the target bovine factor IX (Fig. 6, a and d). In the case of mini-2 aptamer, it could also bind with bovine factor IX but its binding ability was reduced about 4 fold compared with mini-1 (Fig. 6, b and d). Based on the secondary structure of both aptamers it is obvious that the mini-2 lost its stem upon reducing the length and subsequently lost its stability, this could be the probable reason for reduced binding activity. Interestingly,

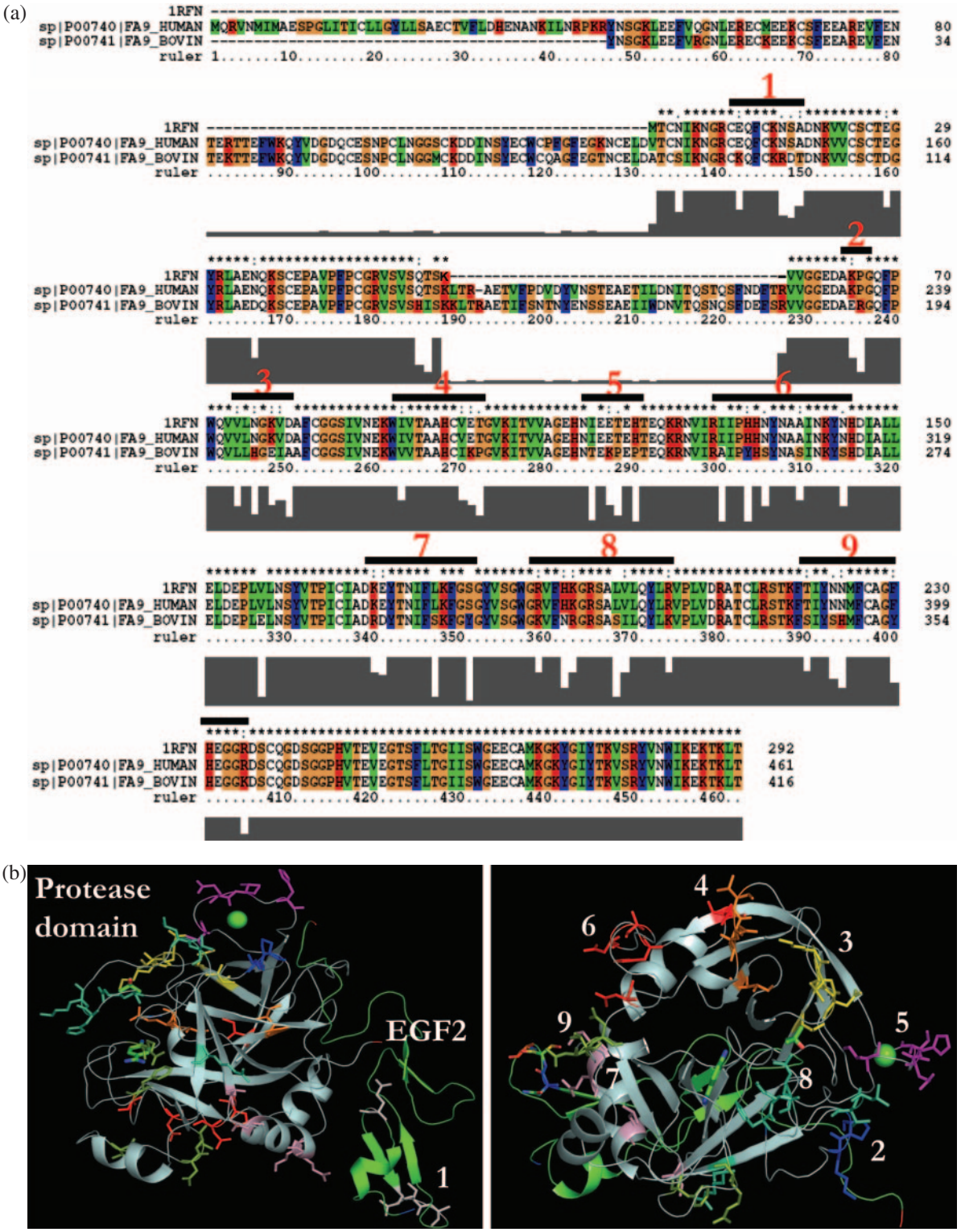

Fig. 7. **(a) Sequence alignment between bovine and human factor IXa.** Shaded regions indicate default ClustalW colouring format for amino acids (GPST, orange; HKR, red; FWY, blue; ILMV, green). Probable binding regions of the selected aptamer in bovine factor IX are indicated by numbers (1–96 to 103 a.a.; 2–189 and 190; 3–198 to 204; 4–217 to 226; 5–239 to 244; 6–254 to 268; 7–294 to 305; 8–313 to 328; 9–344 to 359). **(b) Three dimentional structure of human factor IXa indicated with probable binding sites (shown in different colors) of selected aptamer against bovine factor IX (ribbon models are shown with different angles).** A green sphere indicates a calcium ion.

the mini-3 aptamer with the deleted nts regions from 87 to 92 of mini-1 has completely lost its binding ability to the target, indicating that base groups in the deleted regions are also important to make the complex with bovine factor IX, or that the deletion caused the significant structural rearrangement in the binding region (Fig. 6, c and d).

Since the mini-1 aptamer has high affinity to the target, we prepared the mini-1 aptamer with extended poly(A) tail to check its dissociation constant (K_d) on biacore. The mini-1 aptamer has the similar dissociation constant (~10 nM) as determined for full length. Whereas the complementary sequence of random region has failed to bind with target (Fig. 6e). To determine the discriminating ability of the minimized aptamer we injected with human factor IX at 10 μM concentration and resulting the mini-1 aptamer has failed to bind with human factor IX even at this higher concentration. Based on this study, the discriminating ability of the minimized aptamer is estimated to be more than 1,000 fold.

Probable Binding Site of the Aptamer on Bovine Factor IX—To find the probable binding site of the selected aptamer on the protein bovine factor IX, we evaluated the sequences and structural informations of human and bovine factors. After aligning the sequences from factor IX of both human and bovine, we could locate the differences and we divided these variable regions as 9 segments (Fig. 7a). From the multiple sequence alignment using ClustalW, it was observed that in the EGF2 domain almost all the regions are conserved except near the N-terminus (segment 1) and in the Protease domain 8 variable regions (segments 2 to 9) are observed. In order to understand the arrangement of the variable regions, the three dimensional structure of the human coagulation factor IXa (PDB id: 1RFN) was taken for the analysis. It was observed that the residues near the substrate binding site are well conserved and the probable binding of the aptamer may not be near the substrate binding site. Of these eight variable regions on the protease domain, one region is located near the Ca^{2+} binding site and the other seven are located on the loop regions and exposed to the solvent phase (Fig. 7b). It is generally agreed that the factors from coagulation system are calcium dependent, and calcium ions are essential for proper folding (*21*). The aptamer selected in the present study lost its binding with bovine factor IX when calcium ions are removed from the buffer (Fig. 3c). This indicates that the aptamer binds to the calcium-bound form of factor IX. Moreover, the probable aptamer binding regions on factor IX have accompanied with several positively charged residues which might interact with negatively charged phosphate groups of the aptamer.

Since most of the predicted binding sites of aptamer are located on the protease domain, we checked the proteolytic cleavage of target in the presence and absence of aptamer by our previously established gel-based cleavage assay for the conversion factor IX to IXa with factor XIa (*8*). From this analysis it is clear that the selected aptamer does not interfere in the conversion of factor IX to IXa and it retains the protease activity indicating that the aptamer binding site is far from the active site (Fig. 8). Based on the present and previous studies it is clear that aptamers are capable of binding with high affinity and discriminating among structurally related molecules and it can be concocted in the laboratory. The selected aptamer described here is shown with high affinity and it could discriminate between human and bovine factor IX, this aptamer now joining as a new member in the discriminating aptamer class.

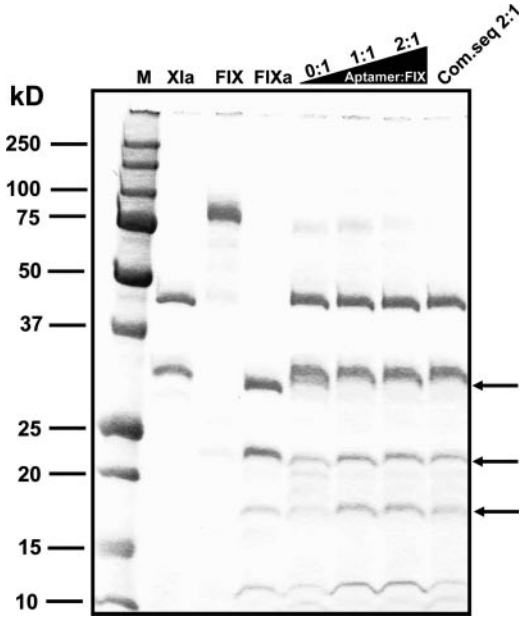

Fig. 8. **Gel-based cleavage assay.** Conversion of factor IX to IXa by a factor XIa was carried out in binding buffer [50 mM HEPES, 1 mM $MgCl_2$, 5 mM $CaCl_2$] with 2.5 μM of bovine factor IX mixed with 620 nM of factor XIa at ambient temperature for 30 min. Similar reactions were also carried out in the presence of the aptamer (1:1 and 2:1 aptamer:bovine factor IX ratio) or its complementary sequence (2:1). For identification, each protein was loaded as a marker. Released products are indicated by arrows.

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