Biochemical and Biophysical Research Communications 457 (2015) 640–646

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

In vitro selection and characterization of deoxyribonucleic acid aptamers against connective tissue growth factor

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ARTICLE INFO

Article history:
Received 22 December 2014
Available online 17 January 2015

Keywords:
Aptamer
Connective tissue growth factor
Systematic evolution of ligands by exponential enrichment
Deoxyribonucleic acid
Dissociation constant

ABSTRACT

Connective tissue growth factor (CTGF) is a secreted matricellular protein possessing complex biological functions. CTGF modulates a number of signaling pathways that are involved in cell adhesion, migration, angiogenesis, myofibroblast activation, extracellular matrix deposition and tissue remodeling. Aptamers are oligonucleic acid chains or polypeptides that bind with specific target molecules hence have the potential to be used in the detection and blockade of the targets. In this study, we selected CTGF-targeting DNA aptamers by using systematic evolution of ligands by exponential enrichment (SELEX). After 8 iterative rounds of selection, cloning, DNA sequencing and affinity determination, six aptamers with high affinities to CTGF were obtained. Among them, one (C-ap17P) binds with the N-terminal region (aa 1-190) and the other five (C-ap11, 12, 14, 15 and 18) bind with the C-terminal region (aa 191-350) of hCTGF specifically. The biological stability assay indicated that a representative aptamer, C-ap17P, could keep its integrity at a rather high level for at least 24 h in complete DMEM cell culture medium. These CTGF aptamers might be used as a easy and fast detection tool for CTGF and be developed as CTGF-specific inhibitors for both research works and clinical applications.

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1. Introduction

Connective tissue growth factor (CTGF/CCN2) is a member of the CCN superfamily of secreted, cysteine-rich glycoproteins [1]. It regulates cell biological behaviors including survival, proliferation, differentiation, migration, adhesion and extracellular matrix (ECM) synthesis by modulating several signaling pathways [2–5]. Studies have shown that CTGF promotes tissue fibrosis by mediating fibroblast proliferation, ECM synthesis, cell adhesion to ECM and epithelial-mesenchymal transition (EMT) [6–8]. CTGF also participates in the development of malignancies by stimulating the proliferation and migration of tumor cells, promoting angiogenesis and modulating the synthesis of extracellular matrix [9–11]. Serum CTGF level is considered to be a potential indicator for the fibrogenic activity and malignant transformation [12]. Moreover, recent studies have demonstrated that blocking CTGF activity can suppress tissue fibrosis and inhibit the development of some cancers [13–15]. Thus, determining the level of CTGF and blocking the bioactivity of CTGF are of significant importance in both research works and future clinical applications.

Specific antibodies are currently the most commonly used tool for the detection and inhibition of secretory molecules. However, in recent years, aptamers have gained more and more attention and are regarded as potential substitutes for antibodies. Aptamers, defined as oligonucleic acid chains or polypeptides folding into specific 3-dimensional structures and binding to specific target molecules, are generated by using an in vitro repeated selection and amplification method known as systematic evolution of ligands by exponential enrichment (SELEX) from a large library of oligonucleotides or polypeptides [16,17]. Except for high affinities and specificities for their targets, aptamers have some prominent advantages over antibodies. For example, the simple SELEX method allows for the obtaining of non-immunological or toxic targets [18,19]; aptamers are synthesized chemically with little batch-to-batch variation, exempting the use of animals or cell lines for the

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http://dx.doi.org/10.1016/j.bbrc.2015.01.041
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production; aptamers can be easily modified to enhance the affinity and stability. Owning to their high specificity, aptamers can be used not only to determine the levels of the target molecules [20] but also to recognize very small structural changes in their target molecules [18]. Moreover, some aptamers bind to the bioactive domains of the target molecules and can inhibit the biological activities [21]. Nowadays, aptamers have found applications in many areas, such as bio-technology, medicine, pharmacology, microbiology and analytical chemistry [22]. An excellent example of the clinical application of nucleotide aptamer is the anti-VEGF165 RNA aptamer (petapganib) which has been successfully used in clinical practice for the treatment of ocular vascular diseases.

In the present study, we obtained six DNA aptamers against human CTGF (hCTGF) by using SELEX. Their affinities to hCTGF were tested and the target regions were preliminarily determined.

2. Materials and methods

2.1. SELEX procedure

Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates were coated with recombinant human CTGF (rhCTGF, ProSpec-Tany TechnoGene Ltd, Ness-Ziona, Israel, 50 ng/well, diluted in a 50 mM carbonate buffer, pH 9.5) overnight at 4 °C and then blocked with 3% BSA dissolved in SHMCK buffer (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, pH 7.4) at 37 °C for 2 h. The starting DNA library (Takara, Dalian, China) was composed of a 40 nucleotide (nt) central randomized sequence flanked on both ends by 18 nt primer hybridization sequences (5′–GACAAGAATCACCGCTC–N40–CGTACAGGAGGCATACAG–3′). The library was dissolved in SHMCK buffer, denatured at 95 °C for 5 min followed by immediate renaturation on an ice-bath for 15 min. Upon selection, 200 ng library DNA was mixed with 10 μg yeast tRNA and 100 μg BSA (both from Sigma–Aldrich, St. Louis, MO, USA) in 100 μL SHMCK buffer and subsequently incubated in a 3% BSA-coated well at 37 °C for 1 h. Next, the supernatant was removed into an rhCTGF-coated well and incubated at 37 °C for 1 h, followed by six washes with SHMCK buffer supplemented with 0.05% Tween-20. Finally, the retained single stand DNA (ssDNA) was eluted with the elution buffer (7 M urea, 0.5 M NH_4Ac, 1 mM EDTA, 0.2% SDS) at 95 °C for 10 min, precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The recovered ssDNA was PCR amplified with the following primers: 5′–GGACAAGAATCACCGCTC–3′ (forward) and 5′–CTGTATGCCTCCTGTACG–3′ (reverse). The 20-cycle PCR was performed by denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s and elongation at 72 °C for 30 s. The agarose gel-purified double strand DNA (dsDNA) PCR product was served as the template to obtain the ssDNA using asymmetric PCR for next round of selection. The 15-cycle asymmetric PCR was performed as described above except that only the forward primer (50 pM) was added. The ssDNA in the asymmetric PCR products was separated by 10% denatured PAGE and extracted using EZ Spin Column PAGE Oligo Gel DNA Extraction Kit (Sangon Biotech, Shanghai, China). The isolated ssDNA was quantified using an $OD_{260/280}$ ratio (Bio-Rad, Hercules, CA, USA) and used for the next round of SELEX.

2.2. Cloning and sequencing

After 8 rounds of selection, the PCR product was ligated into pGEM-T easy vector (Promega, Madision, WI, USA). The ligation product was transformed into *Escherichia coli* DH5α. Fifty colonies were picked and the recombinant plasmids were sequenced. Secondary structures of the inserts were predicted using RNA Structure v3.5.

2.3. Binding assays

The biotin-labeled aptamer candidates with the constant sequences were produced by asymmetric PCR using the 5′-end biotin-labeled forward primer and extracted, denatured and renatured as described above. Subsequently, the recovered biotin-labeled aptamer candidates with constant sequences (100 nM) were incubated in the rhCTGF-coated, 3% BSA-blocked ELISA wells at 37 °C for 1 h. After six washes with SHMCK buffer plus 0.05% Tween-20, the wells were incubated with horseradish peroxidase (HRP) -labeled streptavidin (1:5000 diluted in SHMCK buffer, Cell Signaling Technology, Danvers, MA, USA) at 37 °C for 1 h. Following another six washes with SHMCK buffer plus 0.05% Tween-20, TMB substrate complex was added and the absorbance at a wavelength of 450 nm was measured using a microplate reader (Multiskan MS, Thermo Electron Corporation, Waltham, MA, USA). The test was performed in triplicate wells and repeated for three times. The data were presented as mean ± standard error (SEM). The P values were calculated by student's t test, and $P < 0.05$ was considered statistically significant.

Aptamer blotting assay was used to confirm the binding of the constant sequence-free aptamers to rhCTGF. The 5′- or 3′-end biotin-labeled aptamer sequences were chemically synthesized. rhCTGF was dotted on a nitrocellulose (NC) membrane at 20 ng per spot. An equal quantity of BSA was used as a control. After being blocked with 1% (w/v) human serum albumin in TBSTE (10 mM Tris/HCl, 100 mM NaCl, 0.05 mM EDTA and 0.05% Tween-20, pH 7.0) at 37 °C for 2 h, the membrane was incubated with the renatured biotin-labled aptamers (diluted to 100 nM with TBSE buffer (10 mM Tris/HCl, 100 mM NaCl and 0.05 mM EDTA)) at 37 °C for 1 h. After 3 washes with TBSET, the membrane was incubated with HRP-conjugated streptavidin diluted in TBSET buffer (1:5000) at 37 °C for 1 h. Another 3 washes later, the blots were developed by enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Determination of equilibrium dissociation constant

Equilibrium dissociation constants (K_d) of the aptamers to rhCTGF were determined by measuring the binding of various

Table 1
Aptamer sequence from the selection against rhCTGF, and the table shows the random sequence region only.

Aptamer	Sequence (5′ to 3′)
C-ap1	GGGGAGGGAACCAGGGGCCTTCGGAGACGTCATCATGGGC
C-ap2	CACCAGCACGTTCACGCGGATCTATTCGGTTACGTCCCCC
C-ap3	GGGGCGGGACCTGTACGGGGTAAACAATAGAGGCCGGGGC
C-ap4	CACGAGGTAGGGTACACCGGCCGACATGATAGTCCGTTGC
C-ap5	CACCAATGGACCCACCCTCAGGATCATCCCTCTCGCTGCC
C-ap6	GGCAATGACCCAAGGGAGCCACGGGATCGACGCCCCGCAA
C-ap7	CCCGTCCGCCACCGCAAGCAATCTTACTACCGTCCCGCCC
C-ap8	CACGCCGCCAGGACATCCGTTTATTGCAGTCTCTGCCCCC
C-ap9	CCACGCACCGATAGTAGTCAACCTCAGCACTTGGCGCCCC
C-ap10	CACCCCGGGAGGATAGCTTACCGTCAACCCGTCACCCCCC
C-ap11	GGACAAGAATCACCGCTCCCCGTACAGGAGGCATACAGA
C-ap12	GGACAAGAATCACCGCTCGTACAGGAGGCATACAGA
C-ap13	CTGTATGCCTCCTGTACGGGGAGCGGTGATTCTA
C-ap14	CTGTATGCCTCCTGTACGGGGAGCGGTGATTCTTGTCCA
C-ap15	CTGTATGCCTCCTGTACGAGCGGTGATTCTTGTCCA
C-ap16	AGCGGTGATTCTTGTCCAGCGTGCCTG
C-ap17	AGCGGTGATTCTTGTCCAGCCCTGTATGCCTCCTGTAC
C-ap18	GGCACGTACGCGTCCTGTATGCCTCCTGTACGGGGG

concentrations of the renatured, biotin-labeled aptamers (10 nM, 20 nM, 50 nM, 100 nM, 200 nM, 400 nM, diluted in SHMCK buffer) with a constant amount of rhCTGF (20 ng/well) as described in Section *2.3*. K_d was calculated by fitting the integrated curve of molecule binding using a one-site binding model (hyperbola) in GraphPad Prism software v5.0.

2.5. Determination of the target regions

The coding sequences for the N-terminal (aa 1–190) and C-terminal (aa 191–350) regions of hCTGF were inserted into prokaryotic expression plasmid pET28-a(+), respectively. The hCTGF fragments were expressed in *E. coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography. The target regions of the aptamers were determined by performing binding assay of the biotin-labeled aptamers to the rhCTGF fragments coated on ELISA wells (20 ng/well) as described above.

2.6. Biological stability

The aptamer with constant sequences (200 ng) was incubated in 200 μL Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS at 37 °C under 5% CO_2. Five-microliter samples were taken from the mixture at various time points during incubation. The remaining levels of the aptamer in the mixture were evaluated using PCR followed by PAGE-silver staining.

3. Results

SELEX was initiated with 10^{15} molecules of ssDNA against immobilized rhCTGF. To eliminate non-specific binding and to

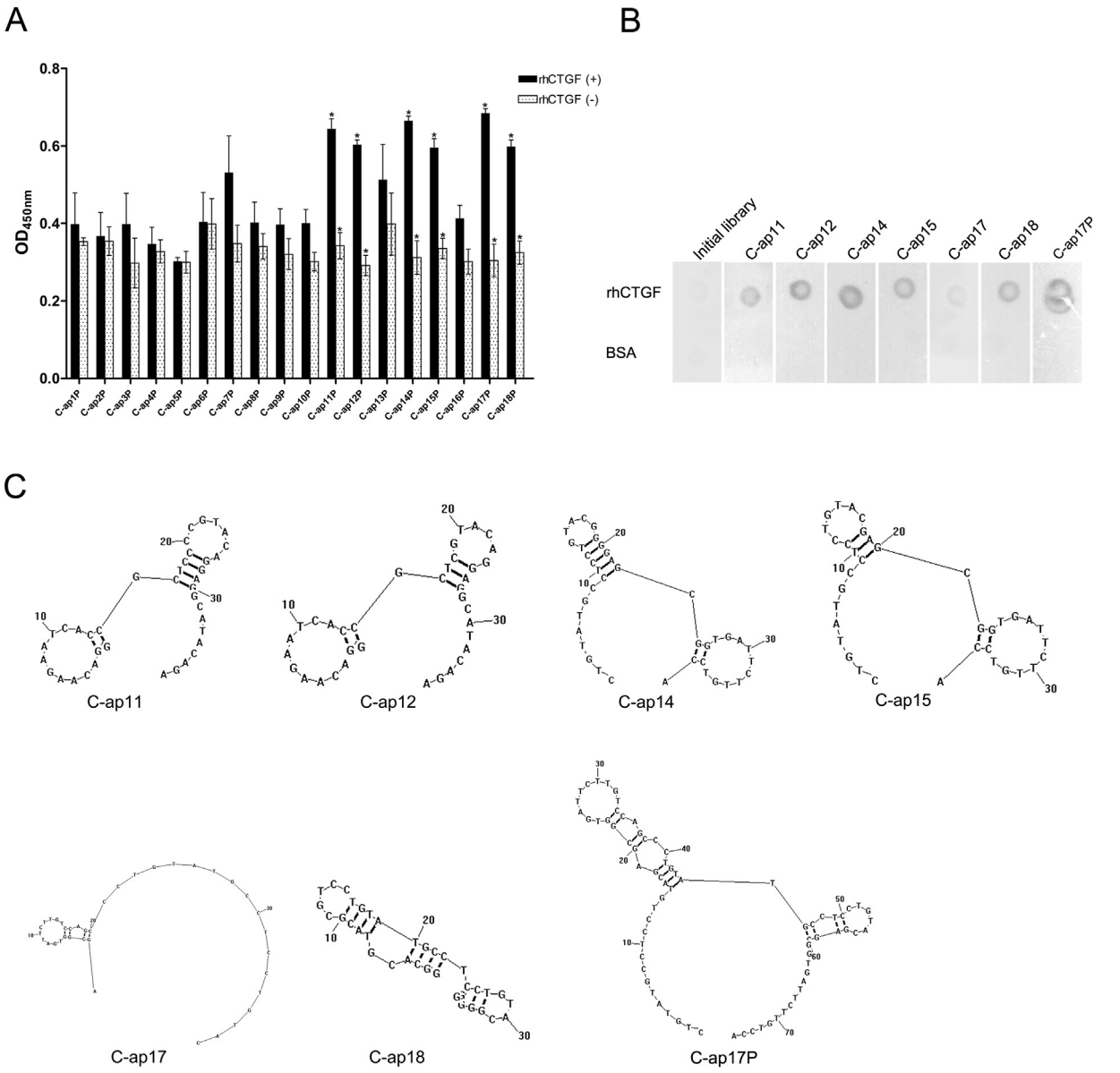

Fig. 1. Selection of DNA aptamers against hCTGF. (A) Affinity of the eighteen aptamer candidates plus constant sequences to ELISA-well bound rhCTGF. BSA-coated wells served as the controls. The affinity was represented as the optical density (OD). The test was performed in triplicate wells and the test repeated for three times (*$P < 0.05$). (B) Specific binding of the aptamers to membrane-immobilized rhCTGF. rhCTGF and BSA were immobilized on the same membrane. The initial aptamer library and BSA served as the controls. (C) The predicted secondary structures of the aptamers.

Table 2
Aptamer sequence binding rhCTGF specially.

Aptamer	Sequence
C-ap11	5′–GGACAAGAATCACCGCTCCCCGTACAGGAGGCATACAGA–biotin–3′
C-ap12	5′–biotin–GGACAAGAATCACCGCTCGTACAGGAGGCATACAGA–3′
C-ap14	5′–biotin–CTGTATGCCTCCTGTACGGGGAGCGGTGATTCTTGTCCA–3′
C-ap15	5′–biotin–CTGTATGCCTCCTGTACGAGCGGTGATTCTTGTCCA–3′
C-ap17	5′–biotin–AGCGGTGATTCTTGTCCAGCCCTGTATGCCTCCTGTAC–3′
C-ap 17P	5′–biotin–CTGTATGCCTCCTGTACG AGCGGTGATTCTTGTCCAGCCCTGTATGCCTCCTGTAC GAGCGGTGATTCTTGTCCA–3′
C-ap18	5′–biotin–GGCACGTACGCGTCCTGTATGCCTCCTGTACGGGGG–3′

increase the selection stringency, a negative selection against BSA prior each round of selection and addition of both yeast tRNA and BSA to the ssDNA solution was persisted throughout the SELEX procedure. After eight rounds of selection, the retained ssDNA was analyzed by cloning and DNA sequencing. After excluding the abundant sequences, eighteen out of the fifty sequences were selected as the aptamer candidates for further analysis (Table 1). Among the eighteen candidates, eight (C-ap11, C-ap12, C-ap13, C-ap14, C-ap15, C-ap16, C-ap17, C-ap18) had the core sequences shorter than 40 nt because of deletions and mutations during the amplification process.

Primarily, the eighteen aptamer candidates with constant sequences were PCR-labeled and their abilities of binding with ELISA well-coated rhCTGF (20 ng/well) were tested. As shown in Fig. 1A,

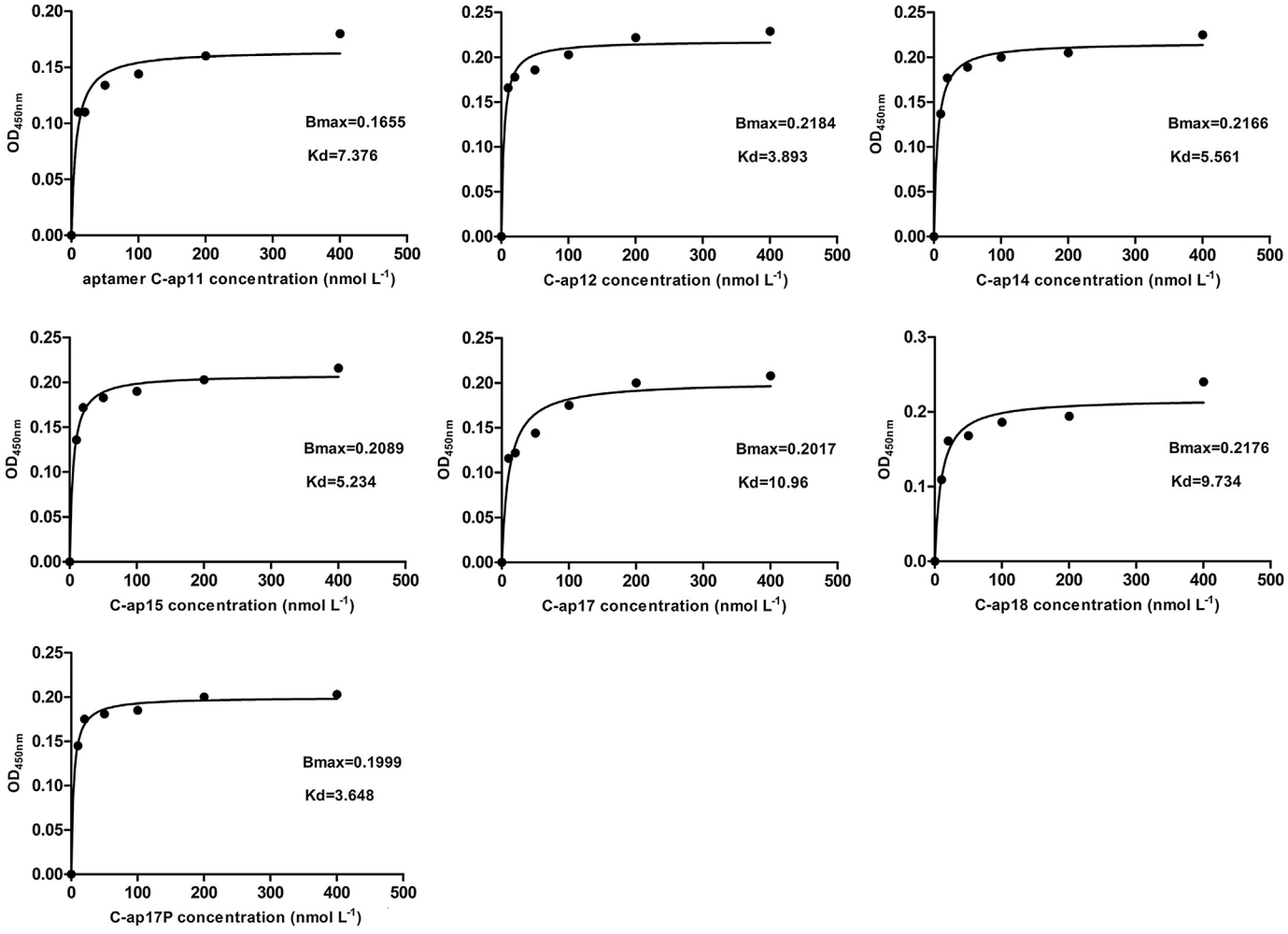

Fig. 2. The equilibrium dissociation constants (K_d) of the aptamers.

six of them (C-ap11, C-ap12, C-ap14, C-ap15, C-ap17, C-ap18) had relatively high affinities to rhCTGF. Next, the core sequences of these candidates were synthesized and labeled with biotin on the 5′ or 3′ end (Table 2). The affinities and specificities of these aptamers to rhCTGF were verified first by measuring their affinities to ELISA well-immobilized rhCTGF and then by aptamer blotting assay. The results showed that all the aptamers hardly reacted with BSA, but bound to rhCTGF with high affinities except C-ap17 (Fig. 1B). Interestingly, we noticed the C-ap17 had an obvious lower affinity to rhCTGF than C-ap17 plus constant sequences (C-ap17P) (Fig. 1B), suggesting that the constant sequences participate in the formation of higher structure for binding to rhCTGF.

We compared the nucleotide sequences of the aptamers and predicted their secondary structures by using RNA Structure v3.5. C-ap11 and C-ap12 share high similarity in sequence. So do C-ap14 and C-ap15. C-ap11, C-ap12, C-ap14, and C-ap15 have a similar predicted secondary structure of two stem-loops linked by a C/G. C-ap11/12 and C-ap14/15 show mirror structures both in the sequence and in the predicted secondary structure. Unlike the above aptamers, C-ap18 has three small stem-loops. C-ap17 has only one stem-loop structure, while C-ap17P could fold into a relatively complex two stem-loop structure (Fig. 1C).

Equilibrium Dissociation constants of the aptamers were determined. As showed in Fig. 2, C-ap11, C-ap12, C-ap14, C-ap15, C-ap17, C-ap17P and C-ap18 bound to CTGF with K_d values of 7.376 nM, 3.893 nM, 5.561 nM, 5.234 nM, 10.96 nM, 3.648 nM and 9.734 nM, respectively.

To determine the respective target regions of the aptamers, the N-terminal (aa 1–190) and C-terminal (aa 191–350) of CTGF were prokaryotically expressed and purified with Ni-NTA chromatography (Fig. 3A). The results of binding tests demonstrated that C-ap11, C-ap12, C-ap14, C-ap15, C-ap18 target the C-terminal region while C-ap17P binds to the N-terminal region of CTGF (Fig. 3B).

The stability of C-ap17P in complete cell culture medium was assessed for the convenience of PCR detection. As shown in Fig. 4, the aptamer could remain in this medium for at least 24 h although the concentration decreased gradually. This result indicates that the aptamer is rather stable in complete DMEM cell culture medium.

4. Discussion

In this study, we obtained six DNA aptamers against hCTGF by using SELEX. The dissociation constants of these aptamers to hCTGF range from 3.648 nM to 9.734 nM, indicating their rather high affinities to the target protein. Moreover, these aptamers target different regions of hCTGF, with one of them (C-ap17P) binding to the 1-190 aa N-terminal region while the rest five (C-ap11, 12, 14, 15, 18) binding to 191-350 aa C-terminal region.

The secondary structure prediction demonstrates that C-ap11, C-ap 12, C-ap 14 and C-ap 15 share high similarity. They all fold into two stem-loop structures linked by a C/G. For C-ap11 and C-ap12, the sequence and secondary structure of the 5′ stem-loop are identical. The difference between them resides in the 3′ stem-loop, in which C-ap11 has three more nucleotides (CCC) rendering a subtle change in this stem-loop structure compared with C-ap12. This change might explain the difference in the affinities. In addition, the different label site might also induce the difference in the affinities between these two aptamers. Moreover, C-ap11/12 and C-ap14/15 contain almost reverse-complementary sequences and show mirror structures. Based on the above analysis, we propose that these four aptamers target a uniform site in hCTGF. This has been partially verified by the result that all these four aptamers target the C-terminal region of hCTGF. C-ap18, although it also binds to the C-terminal region of hCTGF, has a totally different nucleotide sequence and conformation from the above four aptamers. So target site of C-ap18 might be different from the above four aptamers. C-ap17P, which also has a two stem-loop structure, showed a high affinity to the N-terminal region of hCTGF. Interestingly, it will lose binding capacity to hCTGF if the constant sequences are deleted (C-ap17). This phenomenon indicates that a clamp-like conformation containing at least two stem-loops is critical for aptamers because the deletion of the constant sequences will result in the loss of one of the stem-loop structures.

The aptamers obtained in the present study might be used as tools for the detection of hCTGF in biological samples. CTGF is an important and complex molecule composed of four modules: an insulin-like growth factor binding protein module (IGFBP, aa

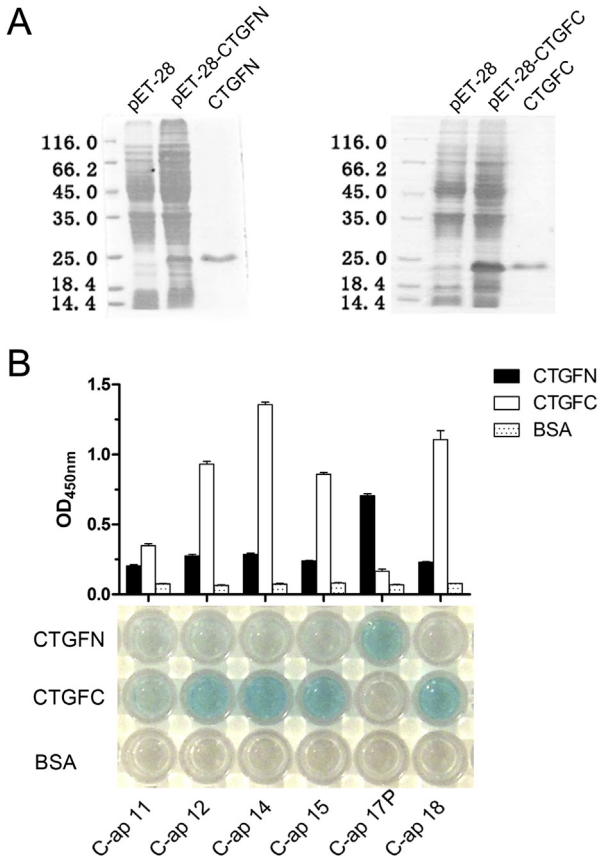

Fig. 3. Determination of the target regions of the aptamers. (A) SDS-PAGE analysis of the prokaryotically expressed and Ni-NTA purified N- and C-terminal regions of hCTGF. (B) The binding of the aptamers to N- and C-terminal regions of hCTGF.

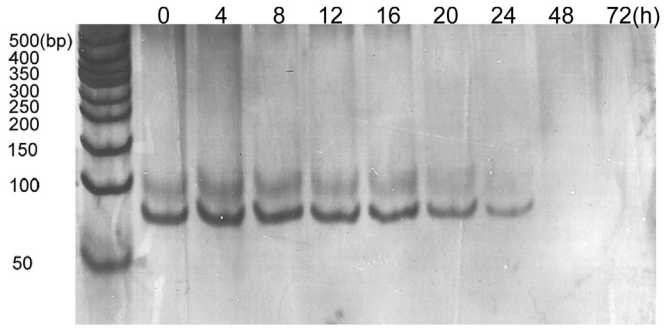

Fig. 4. The stability of C-ap17P in completes DMEM medium. Samples taken from the incubate at 0, 4, 8, 12, 16, 20, 24, 48 and 72 h served as the template to amplify the dsDNA using PCR. The PCR products were analyzed with PAGE following silver staining.

27–97), a von Willebrand factor type C module (VWC, aa 101–167), a thrombospondin type I homology module (TSP, aa 199–243), and a carboxy-termimal cysteine knot motif (aa 256–330). IGFBP and VWC modules comprise the N-terminal domain, which is connected by the hinge region with the C-terminal domain containing TSP modules and cysteine knot motif [23]. Each of the modules might independently exert their own biological functions. Accordingly, CTGF can exist not only in full-length form but also as fragments in serum, urine and extracellular space. Studies have shown that the N-terminal region mainly contributes to myofibroblast differentiation [24] and the C-terminal contributes to fibroblast proliferation [4,24] and Hepatic stellate cells (HSC) activity [25] in tissue fibrosis. Because N-terminal CTGF has a longer half-life in the circulation, its levels in serum are considered as a biomarker for tissue fibrosis rather than the C-terminal or full-length CTGF [26,27]. Apart from fibrosis, CTGF is involved in cancer development and progression by regulating cancer cell migration, invasion, angiogenesis and anoikis [28]. High level of CTGF expression positively correlated with bone metastasis of breast cancer [29], progression of cervical tumors [30], esophageal carcinoma [31] and Wilms' tumor [32], poor prognosis of esophageal adenocancinoma, glioblastoma [33], breast cancer, gastric cancer [34], aggressive behavior and invasiveness of pancreatic cancer cells [35]. Conversely, CTGF expression is negatively correlated with the proliferation activity and tumor grade of chondrosarcoma [36] and non-small-cell lung cancer [37]. These disparities might result from the diversity of CTGF structure, expression level, and binding partners in different tumor types, necessitating the development of more refined detection measures which can differentiate the existence of the different modules and their combinations. The aptamers obtained in this study might fulfill, at least partially, the above needs.

This is only the preliminary results of our study. Before the potential applications of the CTGF aptamers in either laboratory research or clinical practice, there should be a lot of works such as identifying their precise target sites and testing their neutralization abilities. These works are underway at our laboratory presently.

Conflict of interest

None declared.

Acknowledgments

This work was supported by grants from the National Foundation of Natural Sciences, China (No. 81101755 and No. 30871144).

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.041.

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