



Selection of IgE-binding aptameric green fluorescent protein (Ap-GFP) by the ribosome display (RD) platform

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ABSTRACT

GFP-Ck fusion protein was previously shown selectable on ribosome display platform with solid phase antibodies against GFP determinant [Y.-M. Yang, T.J. Barankiewicz, M. He, M. Taussig, S.-S. Chen, Selection of antigenic markers on a GFP-Ck fusion scaffold with high sensitivity by eukaryotic ribosome display, *Biochem. Biophys. Res. Commun.* 359 (2007) 251–257]. Herein, we show that members of aptameric peptide library constructed within the site 6 and site 8/9 loops of GFP of the ribosome display construct are selectable upon binding to the solid phase IgE antigen. An input of 1.0 μ g of the dual site aptameric GFP library exhibiting a diversity of 7.5×10^{11} was transcribed, translated and incubated with solid phase IgE. RT-PCR products were amplified from mRNA of the aptamer-ribosome-mRNA (ARM) complex captured on the solid phase IgE. Clones of aptameric GFP were prepared from RT-PCR product of ARM complex following repetitive selection. Recombinant aptameric GFP proteins from the selected clones bind IgE coated on the 96-well plate, and the binding was abrogated by incubation with soluble human IgE but not human IgG. Selected aptameric GFP proteins also exhibit binding to three different sources of human IgE (IgE PS, BED, and JW8) but not irrelevant proteins. These observations indicate that appropriately selected aptameric GFP on a solid phase ligand by ribosome display may serve as an affinity reagent for blocking reactivity of a biological ligand.

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Ribosome display enables genotype/phenotype linked selection by stalling protein and its cognate mRNA onto ribosomes to facilitate the protein interaction with a solid phase ligand for high-throughput drug discovery [1–4]. Previously, we showed that antigenic determinants on the GFP-Ck scaffold is selectable as an antigen by ribosome display [5]. Herein, aptameric GFP with a combinatorial aptameric peptide library placing in site 6 and site 8/9 is used as an affinity reagent for selective binding to the antigen on the solid phase. RT-PCR product amplified from the mRNA of aptameric protein-ribosome-mRNA (ARM) permits another cycle of transcription and translation, and thus enables recursive rounds of selection for solid phase ligand as a means of drug discovery.

Loop sequences spanning part of the scaffold protein play a critical role in the biological responses. Affinity reagents with aptameric peptide library substituting a native loop on different

protein scaffolds may provide a universal panel of recognition molecules on various protein display platform [6]. Lipocalins and trypsin inhibitor with one loop substitution are forerunner protein scaffolds successfully employed on classical microbial displays [7]. Aptameric peptide library have been constructed into a loop of fibronectin type III domain (FN3), CTLA4, and recently more than one loop are substituted in the ankyrin repeat protein on the ribosome display format [8–10]. GFP is an 11-stranded β -barrel with a coaxial axis, and the ten loops spanned by the 11 β -pleated sheets are exposed to the aqueous phase [11–13]. Among these known protein scaffolds [5–10], GFP is a most thermostable protein with a high melting temperature at $T_m = 82.6^\circ\text{C}$ [14]; moreover, its fluorescence-emitting property renders it a biosensor affinity reagent [15]. Thus, we propose to select IgE antagonists in a combinatorial aptameric GFP library, of which the two loop regions, site 6 and site 8/9 on one hemisphere of the β -barrel are replaced, respectively, with oligonucleotides encoding random decameric peptide library.

Herein, we showed that dual site aptameric GFP, selected by solid phase IgE by ribosome display, bind different human myeloma IgE but not human IgG and other irrelevant proteins. Thus, aptameric GFP-Ck library exhibiting thermostability, solubility and emitting fluorescence may serve as a useful biosensor affinity reagent for the future drug discovery.

Abbreviations: ARM, antibody/aptameric protein; Ribosome, messenger RNA complexes; Ck, constant region of kappa light chain; Rd, ribosome display; FN3, human fibronectin type III domain; GFP, green fluorescent protein; IgE, immunoglobulin E; hulGE, human IgE; hulG, human IgG; Tm, melting temperature.

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Materials and methods

Reagents. Reacti-Bind™ anti-GFP coated plates were purchased from Pierce (Rockford, IL). pGFP_{UV} was purchased from Clontech (Mountain View, CA). Human IgE PS, DES, BED were a generous gift from Professor Hans Spielgelberg at TSRI (La Jolla, CA). Human-murine chimeric anti-NP specific IgE was purchased from Serotec (Raleigh, NC). MAb anti-HA peroxidase conjugate (clone HA-7) and His-Select Nickel affinity gel were purchased from Sigma–Aldrich (St. Louis, MI). Rabbit anti-hulGE HRP conjugate was purchased from PharMingen (La Jolla, CA). INDIA™ HisProbe-HRP, rabbit anti-myc-HRP, anti-GFP coated plates were obtained from Pierce (Rockford, IL). Nickel activated horseradish peroxidase for detecting His-tagged recombinant fusion protein and RT-PCR kits were purchased from Qiagen (Valencia, CA). Hydrophobic tosyl-activated Dynabeads (2.8 µm with *p*-toluene sulfonyl, i.e., tosyl group, 1.3 g/ml) was obtained from Dynal (Dynal, Oslo, Norway). T4 DNA ligase, Vent polymerase and restriction enzymes were purchased from NE Biolab (Ipswich, MA).

Library construction. 5'-PCR1 stuffer was prepared with 5' T7 xb ext, attcaatttgaaactctagaaatagcactcactataggagaccaca acgg and 3' rd4-575r, gtctgccgtgatgtatcac. The 3'-PCR2 with site 6 library was synthesized with the forward primer, Library-f4, gtatacatcacggcagac(NNK)₁₀ggaatcaaaagtaacttc, and reverse primer, ck-r pst aattgtctgcagaccacactcattctgttgaagct. **Site #8/9 library** PCR1 was prepared with 5' T7xb ext and the 3' primer, rd4-688r tggag-tattttgttgataatggc. PCR2 was prepared with library 8-9f, gac-cattatcaacaaaatactcca(NNK)₁₀cattacctgtcgacacaatct and ck-r. PCR1 fragment of combined library was prepared with #6 library as template with T7 xb ext and rd4-688r primer. PCR1 fragment was then annealed with PCR2 with site 8/9 library.

ARM selection of the members that bind to IgE on solid phase. One microgram of DNA prepared from the combinatory library was *in vitro* transcribed and translated at 30 °C for 60 min in TNT Quick Coupled Transcription/Translation Systems (Promega, Madison, WI). Ten micrograms of IgE PS were coupled to hydrophobic tosyl-activated Dynabeads (2.8 µm with *p*-toluene sulfonyl, i.e., tosyl group, 1.3 g/ml) (Dynal, Oslo, Norway), blocked by Tris and washed, resuspended in a 50% slurry. The TNT reaction mixture at 1:8 (v/v) in 120 µl was added to the IgE coupled Dynabeads at r.t. for 45 min, washed, and mRNA eluted from ARM with 10 µl 1× PBS in 20 mM EDTA, and amplified with SensiScript RT-PCR kit (Qiagen) with ck-r pst as a reverse primer, followed by T7 xb ext and ck-r pst primers for 35 cycles (94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min). The aptameric DNA was gel purified for a re-iterative round of selection.

Screening of aptameric GFP binding to hulGE. RT-PCR product from the third round selection was digested with XhoI and SacI, cloned into pGFPuv, modified with N-terminal His-tag and C-terminal myc-tag, and transformed into DH5α. The 96-well plates were coated with IgE PS at 5 µg/ml, blocked and the lysate in 1% Triton added at 1:20 (v/v), or purified protein added from 40 ng/ml to 1 µg/ml, and readout was performed with 1:10,000 dilution of INDIA HisProbe-HRP, which is a nickel activated horseradish peroxidase for detecting the His-tagged recombinant fusion protein, or with 1:5000 dilution of anti-myc-HRP, followed by TMB substrate, and read at OD₆₁₄. Aptameric GFP was prepared by inoculating individual transformed colony in 10 ml LB broth. Pellet was lysed by 1 ml of lysis buffer in 8 M urea, centrifuged at 13,000 rpm. One hundred and eighty microliters of His-Select Nickel affinity gel were then mixed with 1.8 ml lysates, re-equilibrated and renatured with 1.8 ml of 20 mM imidazole at pH 8.0. Protein was eluted in 400 µl of 250 mM imidazole (~100 µg GFP are purified from 10 ml cultures). Binding of purified aptameric GFP was tested

on a 96-well plate coated with hulGE or hulGG, followed by purified recombinant aptameric GFP, HisProbe as described above.

Results

Synthetic single site and combinatory library

Previously we showed the GFP-Ck fusion template is suitable for selection by ribosome display [5]. Herein, single site library with random decamers was placed in the site 6 or site 8/9 of the GFP-Ck template. Moreover, the combinatory site 6 and site 8/9 library was prepared by an assembly PCR with PCR fragments amplified from each respective single site library. As shown in Fig. 1A, PCR2 fragment containing a randomized library of 30 oligonucleotides at the site 6 of GFP-Ck half molecule, was prepared with the library-f4 forward primer and ck-r pst terminal primer. PCR1 stuffer fragment containing the transcription/translation enabling T7 and Kozak sequences was prepared with an overlapping stretch of 18 nucleotides at the 3' end homologous to the 5' sequences of PCR2. Assembly of these two fragments at 1 µg each in a PCR reaction was conducted for 35 cycles without accompanying primers. One microgram of the gel-purified, 1.2 kb fused product (PCR1 + 2, panel i, Fig. 1B) represents a diversity of $\sim 7.5 \times 10^{11}$ of the site 6 library construct.

Moreover, the aptamer library at site 8/9 was constructed with a similar strategy. PCR2 fragment was prepared with a library forward primer, library 8-9f and ck-r pst. A longer 5'-PCR1 stuffer fragment with the overlapping 18 nucleotides at the 3' end, complementary to the 5' of PCR2 was prepared. Fusion of PCR1 and PCR2 fragments at 1 µg each was then conducted by overlap extension PCR for 35 cycles in the absence of primers. Approximately, 1 µg gel purified product of the 1.2 kb fused fragment (PCR 1 + 2, panel ii, Fig. 1B) was obtained, representing also a diversity of $\sim 7.5 \times 10^{11}$ library.

Next, a combinatory library with the above two sites, each with 30 random oligonucleotides, i.e., random decameric peptide for each site, was constructed. In principle, not only numerical diversity is increased up to 5.6×10^{22} (site 6 multiplied by site 8/9) but contact to ligand due to a large surface of combined aptameric library regions may also be improved. As shown in Fig. 1A, the PCR1 fragment with site 6 library was amplified on 1 µg of 1.2 kb single site 6 library as the template for 5 cycles with T7 xb ext and rd4-688r primers. Approximately, 1 µg of the gel-purified PCR 1 fragment was then mixed to the aforementioned PCR 2 construct with site 8/9 diversity region for an extension PCR without the accompanying primers. As shown in Fig. 1B, the assembly PCR yielded a total of 1.4 µg of gel-purified, dual site library construct, representing a actual diversity of 1.05×10^{12} (PCR 1 + 2, panel iii, Fig. 1B) of a potential 10^{22} .

Detection of the aptameric GFP after repetitive rounds of selection

We then determine whether members of the combinatory aptameric GFP library can be selected on IgE coupled solid phase. One microgram of DNA combinatory library was transcribed and translated by TNT T7 Quick system. ARM products, incubated with human IgE-coupled beads, exhibiting pertinent-binding specificities were therefore selected on the ligand-coupled solid phase. As shown in Fig. 2A diagram, following each round of selection, mRNA present on the immobilized ARM, were eluted, amplified by the primer pair, ym-p1, containing the T7 and Kozak sequences and rd4-922r, flanking of the GFP-Ck ribosome display fusion scaffold. Fig. 2B showed that messages eluted from ARM following the third selective round, were amplified as a ~1 kb band (lane 2 of Fig. 2B).

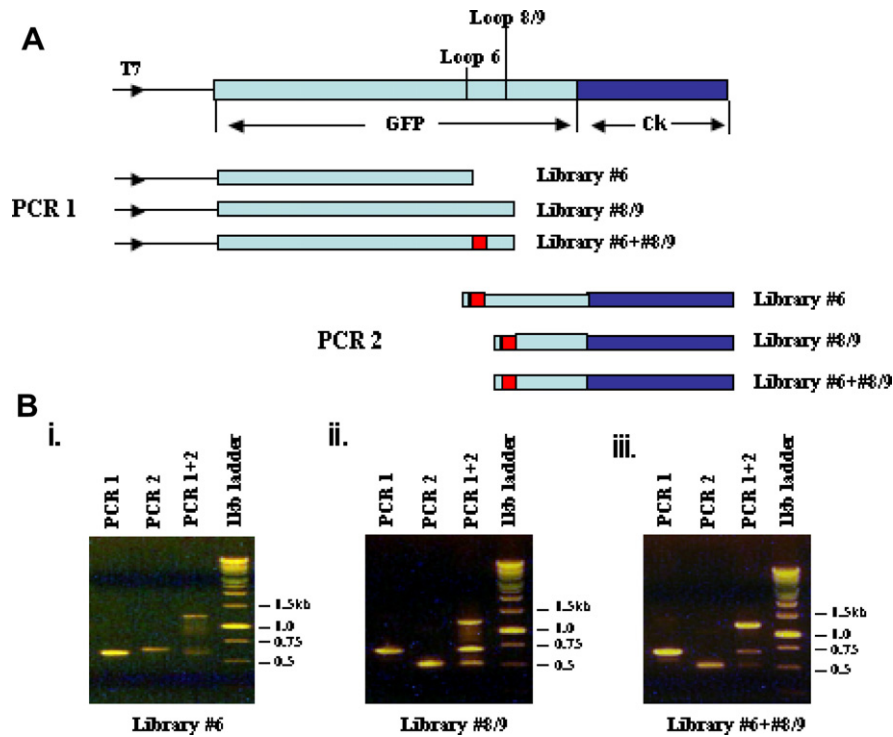


Fig. 1. Construct of a combinatory site 6 and site 8/9 aptameric GFP library. The primers and steps of constructing the single vs. combinatory PCR1 + PCR2 libraries were described in the Material and methods, and Results.

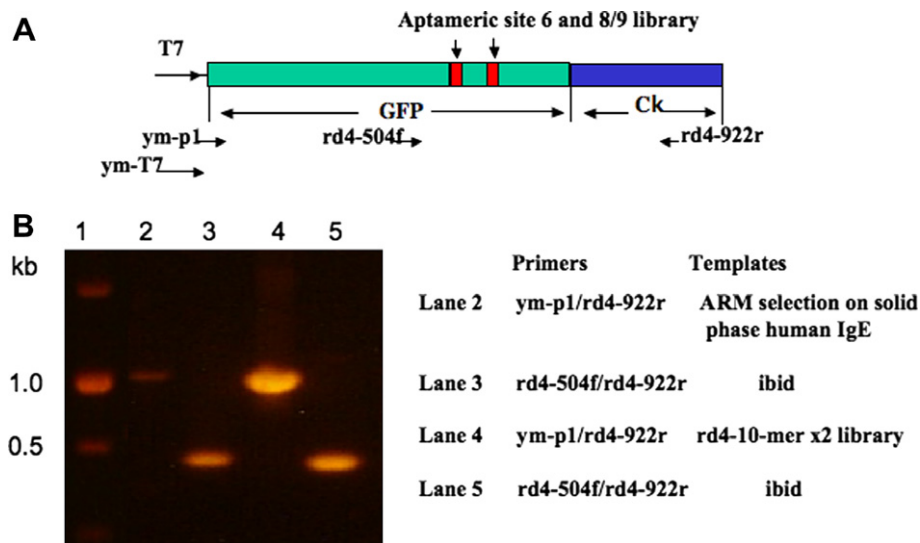


Fig. 2. Amplified RT-PCR products of ARM selected on the solid phase IgE by ribosome display. The use of primers and selection followed by verification of IgE-binding aptameric GFP in each lane were explained in details in the Results.

To validate the authenticity of PCR fragment, the selected messages were also amplified with internal 5' primer, rd4-504f along with the rd4-922r (lane 3 of Fig. 2B). Since this internal 5' primer is streamlined for GC pairing, the shorter ~0.4 kb PCR product appeared more intense than the full length product (lane 3 vs. lane 2, Fig. 2B). Because the internal 5' primer, rd4-504f flanking the downstream critical combinatory library sequences, its choice encompasses the critical selected specificities as well as increases sensitivity for detecting low levels of mRNA due to optimal amplification of a shorter PCR fragment from the selected ARM product. Furthermore, the size of the third round, solid phase ligand-se-

lected, aptameric GFP was corroborated by the similar length corresponding PCR product with the parent library DNA as the template amplified by the terminal primer pair set and/or the internal primer set (lane 4 vs. 2, and lane 5 vs. lane 3, Fig. 2B).

Aptameric GFP-binding specifically to human IgE

To prepare recombinant products for further analysis, RT-PCR products of the mRNA from the selected ARM were subsequently cloned and recombinant protein expressed, purified. The third round selected RT-PCR product (lane 2, Fig. 2B) was digested with

XhoI and SacI, and the recovered fragment containing two aptameric regions were then cloned into similarly digested pGFP_{UV} vector, modified in the laboratory with N-terminus His tag and C-terminus HA tag. The ligated vectors were then transformed into DH5 α , and ~300 individual colonies were picked and grown in 96-well cultures for the subsequent round of an ELISA-based method of screening. To determine the full-length aptameric GFP expression, lysates prepared from the 96-well cultures of the above transformed bacteria, were added, respectively, to the corresponding position of anti-GFP coated 96-well plates, equally divided into two parts, 48 wells each side (Fig. 3A). A full-length recombinant aptameric GFP is anticipated to be positively scored for expressing both N-terminus His-tag and C-terminus myc-tag.

Fig. 3A showed that His-tag was detected with INDIA HisProbe-HRP on the left half of the 96-well (columns 1–6), while Myc-tag was detected with rabbit anti-myc-HRP on the corresponding right half. Except for a relatively higher sensitivity of detection of His-tag than that of the myc probe, both sides of the 96-well plate showed identical scoring pattern. Thus 29 out of 48 were scored positive (twofold above background noise) by INDIA HisProbe,

whereas 28 of 48 were scored positive by rabbit anti-myc-HRP. This indicates that most of the clones express full length GFP following selection by ARM, and expressed as combinatory aptameric library in pGFP_{UV}.

Approximately, close to 20% full length recombinant proteins are scored positive for IgE binding by ELISA. Recombinant proteins of six strong binders were purified by adsorption to the nickel affinity gel. Fig. 3B showed that these six purified aptameric GFP bind to 96-well plates coated with human IgE at 0.2 μ g/ml or 1 μ g/ml, but not to 96-well plates coated with human IgG. Ninety-six-wells coated with hulgE were added with purified clone 2d1 product at 0.4 μ g/ml and incubated concomitantly with hulgE (PS) and hulgG (polyclonal) at increasing concentrations. Purified GFP_{UV} proteins at 0.4 μ g/ml was mixed with increasing concentrations of hulgE or human IgG with recombinant GFP as background controls. Fig. 3C shows that more than 90% binding of recombinant aptameric 2d1 to wells coated with human IgE, were abrogated by co-incubating with human IgE at 10 μ g/ml, subtracting background. In contrast, human IgG added at similar concentrations did not inhibit binding of aptameric 2d1 to solid phase IgE. Table 1 showed that purified aptameric GFP bind all three human IgE: IgE PS, JW8, IgE-DES, but not human IgG. For example, aptameric 2d1 (1 μ g/ml) exhibited ~15-fold higher binding to IgE PS than that to control human IgG (i.e., 2d1, 1.23/0.081). Moreover, Fig. 4 showed that the DNA sequences of these IgE binders from the transformed bacterial colonies are disparate, indicating the difference in the clonal origin.

Discussion

Herein we show, first, a dual site combinatory library containing random aptamers at site 6 and 8/9 of GFP-Ck suitable for ribosome display is constructed by fusion PCR (Fig. 1). Second, appropriately selected aptameric GFP, transcribed and translated from this dual site combinatory library, onto solid phase IgE ligand (Fig. 2), exhibit exquisite-binding specificities for human IgE (Fig. 3) but not with human IgG (Fig. 3 and Table 1). These observations indicate that appropriately constructed aptameric GFP library adaptable on ribosome display platform serves as an *in vitro* high throughput screening platform for studying protein–protein interactions.

Previously we showed that constant antigenic markers on the aptameric GFP-Ck fusion scaffold can be selected by solid phase antibodies at levels of 1–10 femtograms, i.e., as low as input of 750 copies DNA constructs in a single tube reaction according to ribosome display [5]. The diversity of the library is a function of the mass of the input DNA of the ribosome display scaffold when the unique oligonucleotide aptamer inserts, in the form of library primers are provided at the molar excess. Assuming the antigenic reactive group to a particular ligand is at a selectable limit of 750 DNA construct exhibiting a redundant specificity, the aptameric library employed at 10 μ g (7.5×10^{12} molecules for ~1.0 kb DNA construct) will therefore encompass 10^{10} feasible, selectable specificities.

Fig. 2 showed the feasibility of selecting and enriching IgE-binding aptamer present initially in transcribed and translated products of 1 μ g dual site aptameric library. And the recursive round of selection is enabled by subjecting the RT-PCR product, initially amplified from mRNA of the immobilized ARM, to another round of transcription, translation, and the resulting ARM is reselected by solid phase IgE ligand. Harvest of high percentages of human IgE-binding clones (Fig. 3) indicated that recursive rounds of solid phase selection have indeed enriched the relevant-library members. Importantly, full length aptameric GFP were obtained as characterized by reactivities to His and myc at both 5'- and 3'- ends.

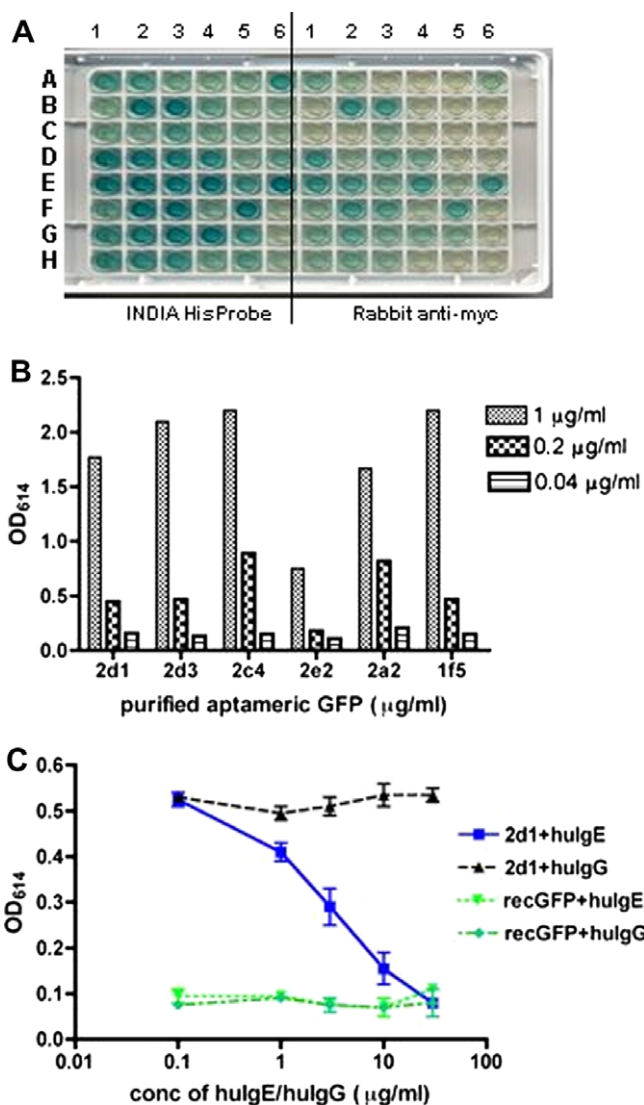


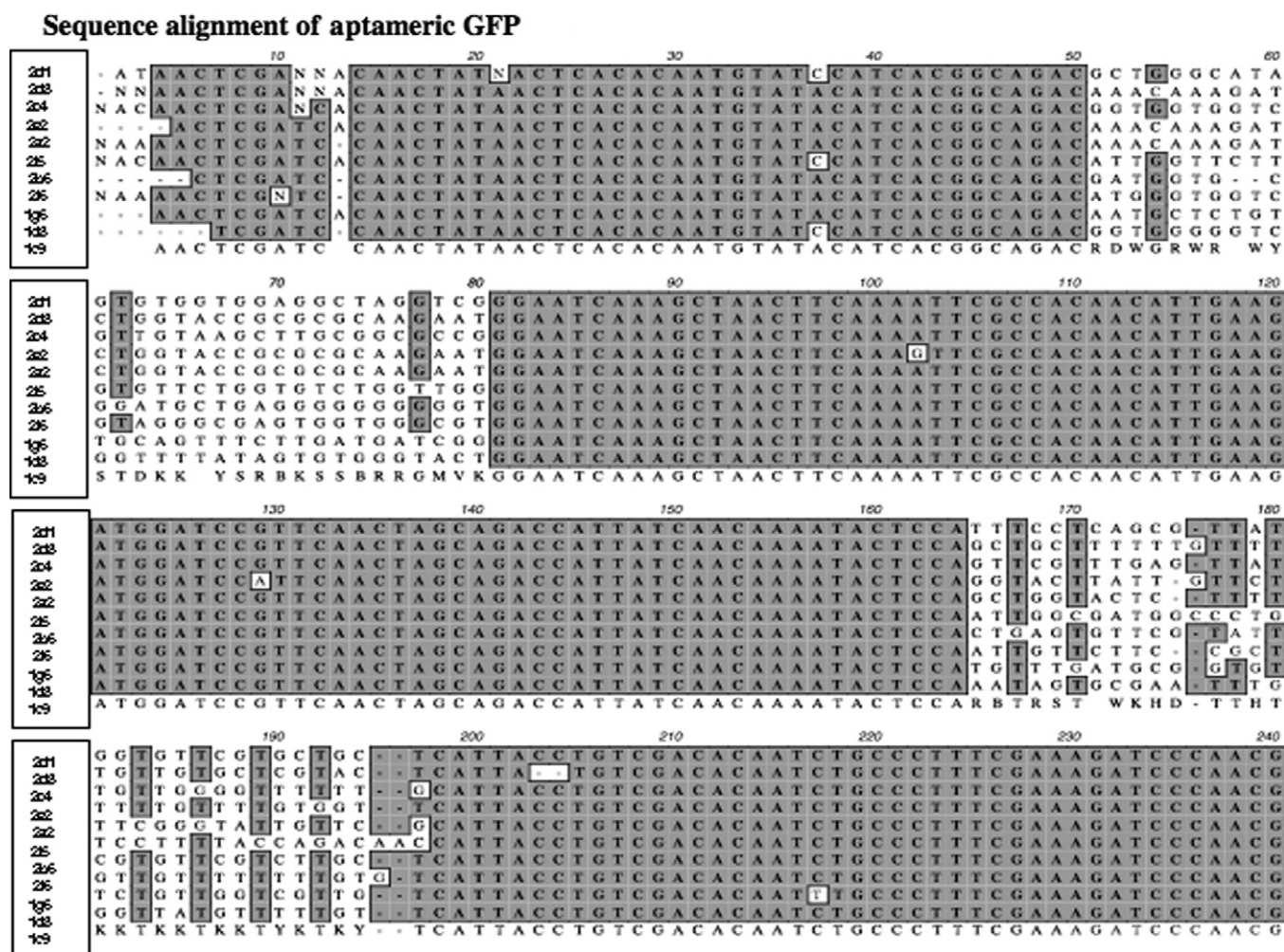
Fig. 3. Validation of IgE binding by recombinant aptameric GFP protein. The procedures and assays were described in details in the Material and methods, and Results.

Table 1

Binding of recombinant aptameric GFP to different human IgE

Aptameric GFP ($\mu\text{g/ml}$)	96-Wells coated with							
	IgE PS		JW8		IgE-DES		HuIgG	
	1	0.2	1	0.2	1	0.2	1	0.2
2d1	1.23	.343	.874	.251	.678	.231	0.081	0.053
2d3	0.905	.268	.979	.292	.743	.165	0.062	.052
2d4	0.833	.242	1.077	.341	.376	.174	0.077	.057
2e2	.399	.104	.197	.141	.453	.221	0.051	.051
2a2	1.164	.262	1.143	.452	.279	.098	0.104	.064
1f5	.978	.28	.849	.267	.167	.34	0.056	.05
GFP _{UV}	.052	.054	.054	.057	.087	.064	.0045	.048
G1 cont	.120	.075	.085	.068	.098	.068	.052	.082

Ninety-six-well plates were coated with 10 $\mu\text{g/ml}$ of proteins, to which nickel affinity gel-purified products were added at 1 $\mu\text{g/ml}$. The plates were washed, added with INDIA HisProbe-HRP, and OD₆₁₄ determined as described in the Material and methods.

**Fig. 4.** Sequence alignment of aptameric GFP. DNA sequences were determined by the Allele Biotech (San Diego, CA).

Thus we conclude the suitability of employing dual site library for selecting full length functional products that exhibit specific-binding activities. Expression of full length aptameric GFP in the cytosol but not the inclusion bodies provides a convenience for protein purification [5].

Thus the two potential advantages of the present dual library design on GFP scaffold are: first, the quality of the contact site formed by the combinatorial merged space may determine the quality of binding, affinity and the exquisite specificities of the reaction. The single chain Fv provides a good quality of spatial

accommodation for ligand, since a random aptamer placed in a CDR will fashion its interactions with foreign ligand along with the remaining CDRs [16]. Choice of protein scaffolds of immunoglobulin supergene family such as FN3 and CTLA4 on ribosome display format were reported with one site replaced with a random oligonucleotide [9,10]. In contrast, the dual library sites (site 6 and site 8/9) within the GFP scaffold are spanned by the adjacent β -pleated sheets around the central β -barrel within the $\sim 697 \text{ \AA}^2$ on one hemisphere with 40 nm depth [11], forming a congruent 3-D combinatorial space comparable to that of a single domain VH

or VL [17]. Thus the dual site library herein may improve the overall quality of contact to solid phase IgE ligand.

Second, GFP is known the most thermostable protein with T_m at 82.6 °C among all known calyx-shaped, β -barrel bearing proteins, including lipocalins [7,14,18,19]. At this temperature, the decimal reduction time is as long as 64 min for quenching 90% of the native fluorescence signal [20]. In contrast, the melting temperature of lipocalins ranges from 44 to 54 °C with natural phosphatidylethanolamine-binding lipocalin (PEBP, T_m = 54 °C), fluorescein-binding lipocalin (T_m = 44 °C) [18,19]. The engineering step apparently lowers the T_m by ~ 10 °C. Although the topological similarity of β -can is shared among the lipocalins and GFP, GFP is far more thermostable than lipocalins by as much as 38 °C. Therefore, GFP may pose an advantage in contrast to lipocalins since the substitution native loops with random aptameric sequences may render the protein scaffold of GFP more heat-labile.

GFP is also favorably compared to another thermostable protein scaffold, fibronectin FN3 (T_m = 78 °C) [8]. The VEGF-binding, engineered FN3 moieties showed the depressed T_m ranging from 50 to 65 °C, reflecting lower stability by a magnitude as much as 28 °C due to engineering [8]. Thus the choice of GFP of a T_m of GFP ~ 82.6 °C may pose as a more robust protein scaffold in contrast to FN3 in addition to being a biosensor. In this context, even with a 20 °C drop in T_m compared to the native GFP, aptameric GFP may still be favorably compared with single domain, camelid VHH exhibiting a T_m around 64 °C [17].

In conclusion, aptameric GFP reactive with the solid phase antigen can be selected from a large repertoire of dual site library constructed within GFP/Ck fusion scaffold by ribosome display. The selected aptameric GFP can serve as a useful affinity reagent for blocking reactivity of a biological ligand such as IgE.

References

- [1] M. He, M.J. Taussig, Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites, *Nucleic Acids Res.* 25 (1997) 5132–5134.
- [2] J. Hanes, A. Pluckthun, In vitro selection and evolution of functional proteins by using ribosome display, *Proc. Natl. Acad. Sci. USA* 94 (1997) 4937–4942.
- [3] A. Rothe, R.J. Hosse, B.E. Power, In vitro display technologies reveal novel biopharmaceutics, *FASEB J.* 20 (2006) 1599–1610.
- [4] M. He, M.J. Taussig, Ribosome display: cell-free protein display technology, *Brief. Funct. Genomic Proteomic* 1 (2002) 204–212.
- [5] Y.-M. Yang, T.J. Barankiewicz, M. He, M. Taussig, S.-S. Chen, Selection of antigenic markers on a GFP-Ck fusion scaffold with high sensitivity by eukaryotic ribosome display, *Biochem. Biophys. Res. Commun.* 359 (2007) 251–257.
- [6] M.J. Taussig et al., ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome, *Nat. Methods* 4 (2007) 13–17.
- [7] A. Skerra, 'Anticalins': a new class of engineered ligand-binding proteins with antibody-like properties, *Mol. Biotechnol.* 74 (2001) 257–275.
- [8] M.H. Parker, Y. Chen, F. Danehy, K. Dufu, J. Ekstrom, E. Getmanova, J. Gokemeijer, L. Xu, D. Lipovsek, Antibody mimics based on human fibronectin type three domain engineered for thermostability and high-affinity binding to vascular endothelial growth factor receptor two, *Protein Eng. Des. Sel.* 18 (2005) 435–444.
- [9] A. Koide, C.W. Bailey, X. Huang, S. Koide, The fibronectin type III domain as a scaffold for novel binding proteins, *J. Mol. Biol.* 284 (1998) 1141–1151.
- [10] G. Coia, L. Pontes-Braz, S.D. Nuttall, P.J. Hudson, R.A. Irving, Panning and selection of proteins using ribosome display, *J. Immunol. Methods* 254 (2001) 191–197.
- [11] M. Ormo, A.B. Cubitt, K. Kallio, L.A. Gross, R.Y. Tsien, S.J. Remington, Crystal structure of the *Aequorea victoria* green fluorescent protein, *Science* 273 (1996) 1392–1395.
- [12] R.Y. Tsien, The green fluorescent protein, *Annu. Rev. Biochem.* 67 (1998) 509–544.
- [13] G.S. Waldo, B.M. Standish, J. Berendzen, T.C. Terwilliger, Rapid protein-folding assay using green fluorescent protein, *Nat. Biotechnol.* 17 (1999) 691–695.
- [14] A. Nagy, A. Malnasi-Csizmadia, B. Somogyi, D. Loroinczy, Thermal stability of chemically denatured green fluorescent protein (GFP) A preliminary study, *Thermochim. Acta* 410 (2004) 161–163.
- [15] C. Kiss, H. Fisher, E. Pesavento, M. Dai, R. Valero, M. Ovecka, R. Nolan, M.L. Phipps, N. Velappan, L. Chasteen, J.S. Martinez, G.S. Waldo, P. Pavlik, A.R.M. Bradbury, Antibody binding loop insertions as diversity elements, *Nucleic Acids Res.* 34 (2006) e132.
- [16] R. Billetta, M.R. Hollingdale, M. Zanetti, Immunogenicity of an engineered internal image antibody, *Proc. Natl. Acad. Sci. USA* 88 (1991) 4713–4717.
- [17] S. Ewert, C. Cambillau, K. Conrath, A. Pluckthun, Biophysical properties of camelid V(HH) domains compared to those of human V(H)3 domains, *Biochemistry* 41 (2002) 3628–3636.
- [18] J. Wiedersich, S. Kohler, A. Skerra, J. Friedrich, Temperature and pressure dependence of protein stability: The engineered fluorescein-binding lipocalin Flua shows an elliptic phase diagram, *Proc. Natl. Acad. Sci. USA* 105 (2008) 5756–5761.
- [19] B. Vallee, C. Teyssier, R. Maget-Dana, J. Ramstein, N. Bureaud, F. Schoentgen, Stability and physicochemical properties of the bovine brain phosphatidylethanolamine-binding protein, *Eur. J. Biochem.* 266 (1999) 40–52.
- [20] T.C. Vessoni Penna, M. Ishii, O. Cholewa, L.C. de Souza, Thermal characteristics of recombinant green fluorescent protein (GFPuv) extracted from *Escherichia coli*, *Lett. Appl. Microbiol.* 38 (2004) 135–139.