Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 465–473

BIOORGANIC & MEDICINAL CHEMISTRY

DNA-Binding Peptides Searched from the Solid-Phase Combinatorial Library with the Use of the Magnetic Beads Attaching the Target Duplex DNA

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Received 12 October 1999; accepted 16 November 1999

Abstract—We have exhibited successful and rapid screening of DNA-binding peptide ligands from solid-phase library beads with the use of the target DNA-conjugated magnetic beads. The target duplex DNA (**3**) has a polyether linker between two complementary sequences (T_4A_3G-ether linker-CT_3A_4) and is stable in the duplex form during the selection procedure. Finally, 71 pentapeptide sequences were identified from the solid-phase pentapeptide library. From an analysis of the peptide sequences identified in this study, it has been revealed that peptide ligands contain hydrophobic amino acids as the major component. The synthetic peptides with identified sequences and a combination of the major components have exhibited moderate to high binding affinity to the duplex DNA in competition experiments with ethidium–DNA complexes.

Introduction

It is now widely accepted that most diseases contain genetic disorder. Therefore, development of new therapeutic methods for sequence-selective inhibition of disordered genes is highly desired. A number of approaches have been attempted with the use of oligonucleotides-based inhibitors, DNA-binding ligands with small molecular weight such as naturally occurring substances, etc.[1–6] Oligonucleotides and related compounds can recognize a duplex DNA sequence within the major groove by forming triplexes.[7–12] However, duplex sequences suitable for triplex formation are limited only to homopurine–hornopyrimidine tracts. Recently, polyamides as the analogues of natural products have been shown to recognize a DNA sequence within a minor groove.[13–19] Nevertheless, accurate recognition of a DNA sequence has remained a difficult problem. In this study, we have attempted to find short peptide ligands for the target duplex DNA based on combinatorial technology. Binding motifs of DNA-binding proteins consist of relatively short peptides, and their binding properties are originated from the fixed conformation of a protein backbone.[20] Determination of peptide ligands by random screening and their structure analysis would provide useful information on small molecular ligands for DNA-binding. In this paper, we wish to report the following: (a) random screening of new peptide ligands from the solid-phase pentapeptide library by combinatorial technology, and (b) quantitative analysis of the binding affinity of selected pentapeptides with the duplex.

Structures of the Library and the Magnetic Beads, and the Screening Method

The chemical structures of the library and the magnetic beads are schematically shown in Figure 1, and Figure 2 illustrates the screening procedure. The library beads were constructed with Merrifield resin by pool-and-split synthesis, incorporating one sequence per (one) bead.[21] Two units of 6-amionocaproic acid were first incorporated into the resin as the spacer, followed by elongation of pentapeptide by conventional solid-phase synthesis with the use of 20 *N*-Fmoc-protected *L*-amino acids to provide library beads (**1**) containing 3,200,000 sequences (20^5) in total. We have already reported a combinatorial random screening method with the use of magnetic beads conjugated with the target molecule and the library beads.[22] In this study, we attempted to apply this method to search for peptide ligands toward the target duplex DNA that is attached on the magnetic beads (**2**).[23,24] The sequence of the target duplex DNA (**3**) (T_4A_3G-ether linker-CT_3A_4) was chosen as a model

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PII: S0968-0896(99)00298-9

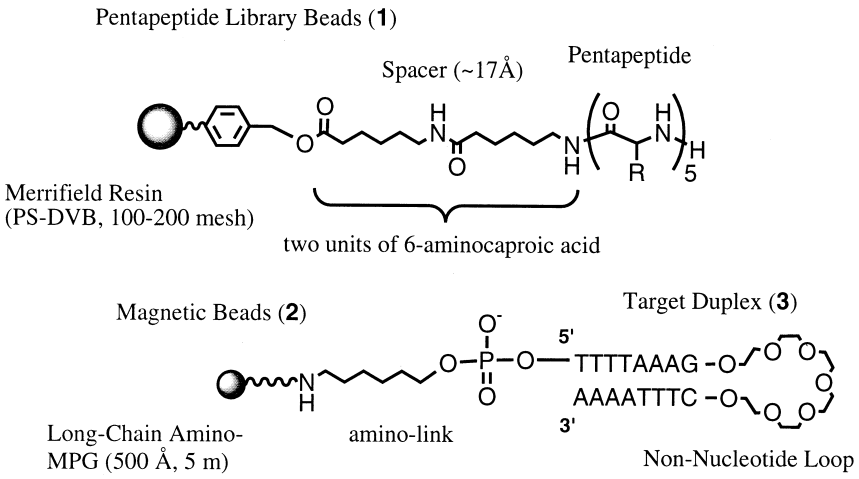


Figure 1. The structures of the library (**1**) and the magnetic beads (**2**).

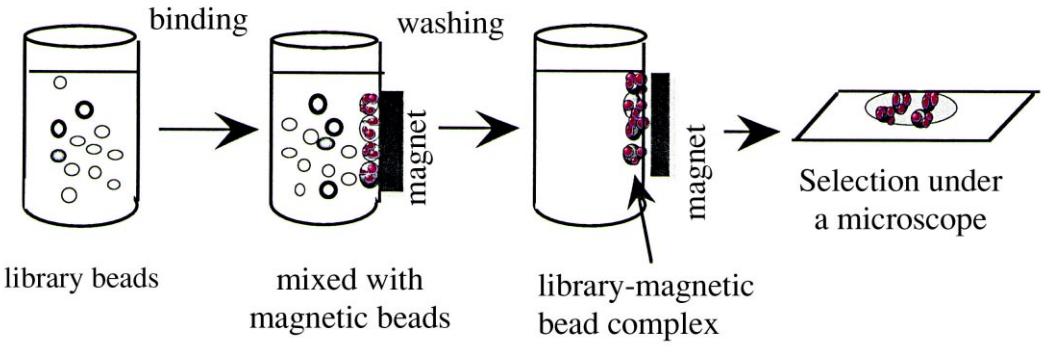


Figure 2. Schematic procedure of random screeninc, using, the library and magnetic beads.

duplex. The target duplex DNA (**3**) forms a stable duplex (T_m = 52–54 °C) due to an ether linker as a non-nucleotide loop, suggesting that the duplex structure is maintained during the screening process at room temperature. The target duplex DNA (**3**) was synthesized bearing an amino-linker at the 5′ end, and attached covalently to the magnetic beads by a reductive amination procedure.

A mixture of the library (**1**) and the magnetic beads (**2**) in a buffer was gently shaken in a plastic tube for 3 h at room temperature. A strong magnet was placed outside the wall of the tube. The interacting bead–bead complexes adhered to the inside wall of the tube at the position of the outer magnet. Interacting beads were collected, and observed to be attached by a number of magnetic beads on the surface (Fig. 3). Such library beads were separated by a fine glass capillary under a microscope, and subjected to a peptide sequencer for determination of the sequence.

Synthesis of the magnetic beads connecting duplex DNA

The target duplex DNA (T_4A_3G-ether linker-CT_3A_4, **3**) was synthesized by an automated synthesizer using the DMTr-protected amidite precursors of 2′-deoxynucleosides of adenosine, thymidine, guanosine, and cytidine. A polyether spacer as a non-nucleotide loop. (DMTrO(CH_2CH_6O),OP(N*i*Pr_2)O–CH_2CH_2CN) was introduced between the two complementary strands of DNA.[25] At the final step of the synthesis, aminolink2 (TfNH$(CH_2)_6$OP(N*i*Pr_2)O–CH_2CH_2CN) was introduced to the 5′ end, followed by cleavage with 28% NH_4OH. The crude oligomer was purified by reverse-phase HPLC to give the aminolink-conjugated target duplex (**3**). Melting temperature of the target duplex (**3**) ranged from 53 to 56 °C.

Next, the aminolink-conjugated target duplex (**3**) was attached to the magnetic beads (MPG long chain alkylamine, 500A-5 micron) according to the conventional protocol of reductive amination with the use of glutaraldehyde and $NaCNBH_3$ (Fig. 4). A part of the magnetic beads (**2**) was hydrolyzed with venom phosphodiesterase (VPDE) and bacterial alkaline phosphatase (BAP); then the supernatant was analyzed by reverse-phase HPLC. Four nucleoside components were observed in the almost expected ratio of A:T:G:C = 7:7:1:1, confirming successful introduction of the target duplex (**3**) on the magnetic beads (Fig. 4).

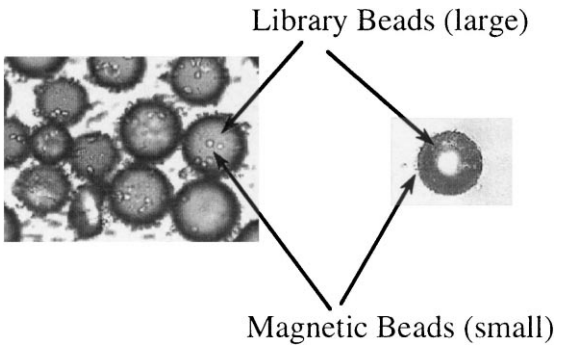


Figure 3. Microphotograph of complexes between library and magnetic beads (×200 magnification).

Results

Determination of sequences of the selected beads

Table 1 summarizes the 71 sequences of pentapeptides on the beads selected by the random screening. Unidentified — amino acids were indicated by X, some of which may be *t*Bu-protected cystein. The name and the number of amino acids identified at each position of the pentapeptide sequence are summarized separately in Figure 5, illustrating only those amino acids identified in more than five peptides. Phenylalanine (Phe; F) and asparagine (Asn; N) were determined most frequently at the N-terminal position. Glutamine (Glu; Q) was the best amino acid contained in the second residue. Tyrosine (Tyr; Y), glycine (Gly; G), tryptophan (Try; W) and phenylalanine (Phe; F) were determined as the preferable amino acid for the third amino acid. Isoleucine (IIe; I) was favorable at the fourth position, and tyrosine, isoleucine (IIe; I) and leucine (Leu; L) were determined frequently at the fifth residue. Totally, tyrosine (Tyr; Y), phenylalanine (Phe; F), isoleucine (IIe; I), leucine (Leu; L) and asparagine (Asn; N) were determined most in the pentapeptides. Interestingly, it turned out that hydrophobic amino acids are the most common components of the pentapeptides that were selected in this random screening. On the other hand, it should be noted that no basic or acidic amino acids were determined as the most frequently appearing amino acids. Also, some amino acids were completely excluded from a specific position in the pentapeptide sequences, for example, glutamine in the first residue, serine and alanine in the second, serine and threonine in the third and glycine in the fifth position. Therefore, we assumed that peptides constructed of the most frequently appearing amino acids at each position might have a high affinity to DNA.

Table 1. Pentapeptide sequences determined by the random screening[a]

EPFSF	GYEAN	NXMMR
FTGVM	IXWHX	DYVHI
XFNDW	IRMYI	SVLGL
FTNNP	FYFIS	TNYDX
KVIEX	RLPYM	NYXEX
XLYYF	LQQPL	FWWII
FWGHE	YLIAN	DIMEX
TEHQL	MQXVY	IDARX
FMYIR	TGFGW	AHNLA
PDADH	GFKPF	XQGDX
ADYLH	FLDWW	NKYYY
DXGIX	GVQXV	NMWYV
AQNRA	EYFRV	DKXXY
FDWXX	HXMXX	YXVFL
MGPFF	AQYXI	WYHIY
IWLXX	LDWAA	NQYXX
NRGII	LQMXR	XQXXX
TLQLA	LFNMX	YVVKX
NTIIL	TFFGX	PFRPP
EDVHX	VTWSX	VKPXX
GPHNQ	KGFTD	NEIGX
INGGY	KTIKA	ANREL
ILPEI	YFXRT	KXLXP
RWXHX	VQHXY	

[a]X, not identified.

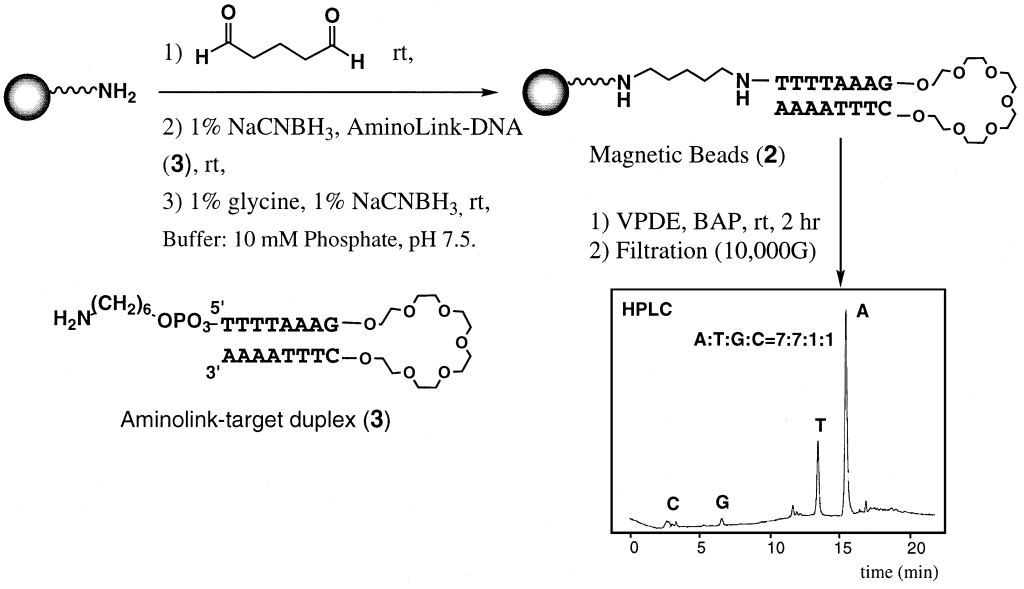


Figure 4. Preparation of the magnetic beads—connecting the target DNA **3**, and analysis of enzymatic hydrolysate of the magnetic beads (**2**).

Evaluation of binding affinities of pentapeptides by ethidium displacement assay

Binding affinity of the peptide ligands with the duplex DNA was estimated by the displacement of DNA-bound ethidium bromide (ETBr). The emission intensity of ethidium bromide is increased by binding with duplex DNA, and is inhibited by competition with another DNA-ligand. The binding of ethidium bromide with DNA contains several binding modes, and also inhibition of emission of ethidium bromide does not reflect the binding mode of the competitive ligand.[26–32] Thus, the ethidium displacement assay is convenient when the binding mode of the ligand is not known. In this study, only selected pentapeptides and model DNA duplexes (**3**, A3T3, CA12, CT12) were used for measurement of binding affinity (Table 2). The target duplex (**3**) and self-complementary 12-mer duplexes (A3T3 and CA12) consist of a different number of AT pairs at the center part of the duplex. CT12 has four GC pairs at the

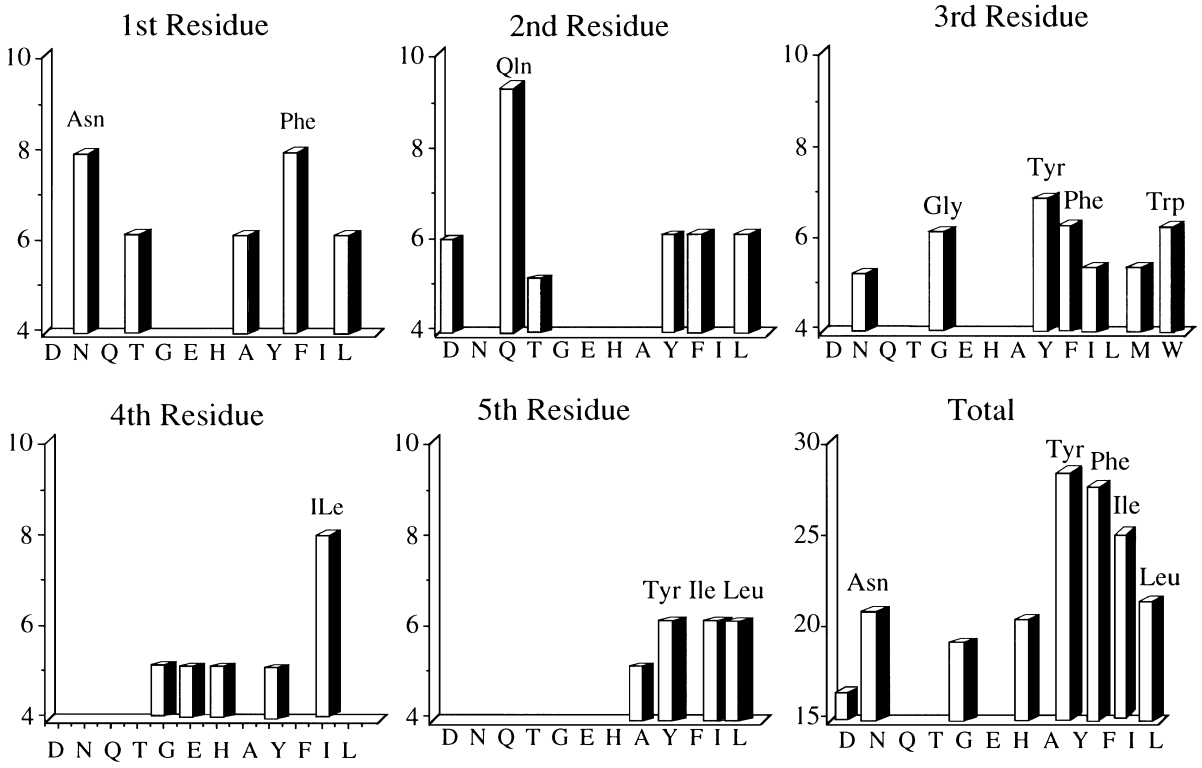
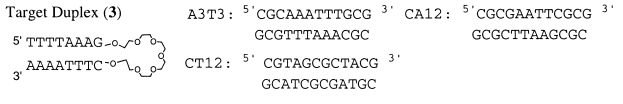


Figure 5. The number of amino acids determined at each position of pentapeptides. Only those contained in more than five peptides are shown.

Table 2. Binding affinity of peptide ligands[a] (C_{50}, μM)

Entry	Peptides	Target duplex (**3**) $K_{ETBr} = 3.9\times10^6$	A3T3 $K_{ETBr} = 2.5\times10^6$	CA12 $K_{ETBr} = 7.6\times10^6$	CT12 $K_{ETBr} = 2.4\times10^6$
1	FQGII	7.2	4.2	7.8	2.8
2	FQWII	24.5	34	11	10
3	FQFII	22	15.6	25	12.5
4	FQYII	35	17.5	45	17
5	WATTT	19	22	19	12
6	NQGIL	36	33	17.8	11.8
7	ILPEI	9.3	7.2	11.6	4.9
8	AHNLA	8	9	14.5	6
9	KTIKA	24	9	24.5	7.5
10	EPFSF	14.5	10.5	23	10
11	FTGVM	27	15.7	34.3	12.2
12	TEHQL[b]	14.3	17.2	20	8
13	KGFTD	37	37.5	24	10
14[c]	FQWII	1.4×10^6	0.2×10^6	1.0×10^6	1.9×10^6
15	Distamycin	—	—	No inhibition	19

[a]DNA as duplex = ethidium bromide = 1.0 μM, 10.0 mM SHE buffer, pH 5.0, Ex = 546 nm, Em = 595 nm.
[b]No caproic acid unit connected to C-terminal.
[c]Binding constants (K_s, M^{-1}) were determined directly by titration experiments under the same condition.



middle of the duplex. First of all, we synthesized pentapeptides, FQ-X-II (X = G, W, F, or Y) and NQGIL which are composed of amino acids that were identified most frequently at each position. The sequences ILPEI, AHNLA, KTIKA, EPFSF, FTGVM, TEHQL and KGFTD were found in the sequences of some DNA or RNA binding proteins, and were chosen for binding assay, although it is uncertain whether these sequences are involved in the binding sites of the proteins. The sequence of WATTT contains amino acids that were not found in this assay. All of these peptides except for TEHQL, contain two units of 6-aminocaproic acid at the C-terminal.

Prior to the ethidium displacement assay, we determined the binding parameters of ETBr–DNA binding. Emission intensity of ETBr was measured in the function of the duplex DNA concentration. Figure 6 illustrates job plot and titration experiments with ETBr and the 12-mer duplex (A3T3) at 25 °C, showing a ratio of ethidium:DNA = 2:1 with a stability constant of $K_s = 2.5\times10^6$ M^{-1}.[33] Binding constants of ETBr to the other duplexes were also similarly obtained, and are shown in Table 2.

As binding stoichiometry of the pentapeptides could not be determined, the binding affinities of the peptides were compared by determining the concentration needed for 50% decrease of emission intensity of ETBr (C_{50}, μM) (Table 2). As the tryptophane-containing peptide (FQWII) is fluorescent, the binding parameters could be obtained directly.[34–38] In this case, the intensity of FQWII was decreased by binding with the DNA. The job plot and titration experiment confirmed 1:1 complexation with the binding constants listed at entry 14 in Table 2.

The sequence of FQGII is a simple combination of amino acids with the highest appearance at each position. This peptide showed the lowest C_{50} values toward all sequences of DNA, indicating the highest affinity compared to other peptide sequences (Table 2, Entry 1). The binding affinity of FQGII is higher than that of distamycin under the same condition (Entries 1 versus 15). In contrast, FQ-Y-II, FQ-W-II and FQ-F-II with different amino residue at the third position exhibited lower affinity to DNA compared to FQGII (Table 2, Entries 2–4). Binding affinities of FQWII were obtained directly by monitoring tryptophan fluorescence quenching, and somewhat lower affinities to all duplex DNA than that of ETBr were obtained. Based on the comparison with FQWII, FQGII is estimated to have, at least, comparable binding affinity with ETBr. Displacement of only one amino residue from G to W, F or Y caused a remarkable decrease of binding affinity (Entries 1 versus 2, 3, 4). The sequences WATTT and NQGIL showed a relatively moderate binding affinity to all duplexes (Entries 5 and 6). Among the other peptides, ILPEI and AHNLA showed a relatively high affinity (Entries 7 and 8).

The C_{5O} values of FQ-X-II (X = G, W, F, Y) are plotted against the duplex DNA sequence (Fig. 7). The binding affinity of ethidium bromide (ETBr) is also plotted for comparison. FQGII exhibited the highest affinity to all sequences nonselectively, similar to ETBr. Change of one amino acid in FQ-X-II showed little effect on the bindings to CT 12, but induced a different effect to the other DNA sequences. Binding of FQFII or FQYII became much weaker than that of FQGII toward CA12; FQWII was the weakest ligand to A3T3; peptides, except for FQGII, showed only low affinity to the target duplex DNA (**3**). As a result, FQWII showed selectivity to CA12 and CT12, and FQYII became selective to A3T3 and CT12.

Discussion

In this study, we have applied combinatorial technology to search for peptide ligands toward duplex DNA with the use of the solid-phase library and the magnetic beads conjugating the target duplex DNA. As a result, 71 pentapeptides were determined, and a clear tendency was obtained in that only neutral and hydrophobic amino acids were identified in the selected pentapeptides and no basic or acidic amino acids were determined (Table 1 and Fig. 5). Some pentapeptides of the determined sequences

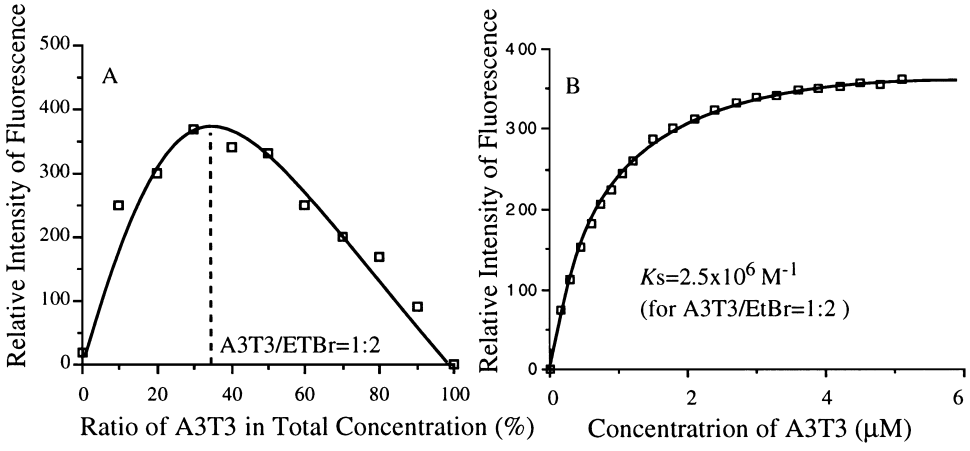


Figure 6. Determination of binding parameters of ethidium bromide (ETBr)-A3T3 complexation: (A) job plot indicates A3T3:ETBr = 1:2 complexation: (B) theoretical curve was obtained by assuming A3T3:ETBr = 1:2 complexation with $K_s = 2.5\times10^6$ M^{-1}.

were synthesized and their binding affinities were estimated by ethidium displacement assay (Table 2). It was of great interest that the FQGII, a simple assembly of the most-frequently-appeared amino acids at each position, showed the highest affinity to all duplex DNAs examined here. From these results, a clear conclusion has been obtained; the hydrophobic nature of small molecules is one of the important forces for binding with DNA. This finding is also consistent with other results with hydrophobic compounds.[27,28,39] As DNA and RNA possess a highly negatively charged phosphate backbone and the nucleic acid binding sites of these proteins possess a positively charged amino acid, it is generally understood that the stabilities of most protein-nucleic acid complexes receive large contributions from electrostatic interactions.[40–42] Binding poly(U) of polycationic peptides such as RWRRRR-NH_2 or KWKKKK-NH_2 has been investigated, and binding constants ranging from 10^3 to 10^5 were obtained.[43] The peptides identified in this study, including FQWII, have one positive charge at the N-free terminal and one negative charge of the carboxyl group at the C-terminal, having no net charge. Nevertheless, these peptides showed comparable or somewhat stronger binding affinity to the duplex DNA compared to the binding affinity of the above polycationic peptides. This comparison again suggests that the hydrophobic structure plays an important role for binding of small molecules to duplex DNA. It has recently been reported that specific binding of Hoechst 33258 to the A3T3 duplex is mainly driven by hydrophobic interaction rather than H-bonding or van der Waals contacts.[44] Although we could not obtain general information about the peptide structure for selective binding to a specific DNA, it is interesting that change of one amino residue of pentapeptide (FQ-X-II) had a different effect on the DNA binding and produced some selectivity (Fig. 7). FQGII probably has a flexible structure because of a small glycine residue, and may change its conformation for binding to duplex DNA, causing nonselective binding. The conformation of FQ-X-II may be restricted by a relatively large aromatic residue of Y, W or F, thus producing some selectivity. More detailed experiments will be needed for further accurate analysis of the binding properties of the peptide ligands.

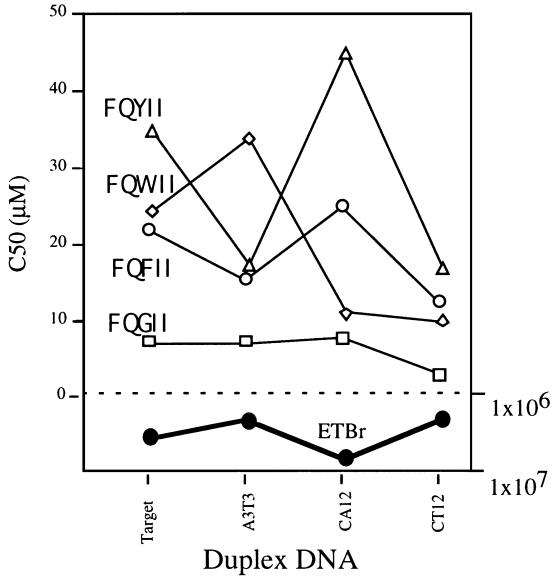


Figure 7. Sequence selectivity of pentapeptides. The left axis represents the C_{50} (μM), and the right axis represents K_s (M^{-1}) of ethidium bromide (ETBr).

Conclusion

In conclusion, we have exhibited successful and rapid screening of DNA-binding peptide ligands from library beads with use of the target DNA-conjugated magnetic beads. From the analysis of peptide sequences identified in this study, it has been revealed that peptide ligands contain hydrophobic amino acids as the major component. The synthetic peptides with identified sequences and a combination of the major components have exhibited moderate to high binding affinity to the duplex DNA. This study has clearly suggested that hydrophobic interaction may be a major contributor for binding of small molecules to duplex DNA.

Experimental

Synthesis of the target duplex DNA (3) conjugated with aminolink

The target duplex (aminolink-T_4A_3G-polyether linker-CT_3A_4) was synthesized with the automated DNA synthesizer (CyclonePlus DNA synthesizer, MilliGen/Bioresearch) on a CPG resin by the standard phosphoroamidite chemistry. The amidite precursors of 2′-deoxynucleotides were purchased from Glen Research, and DMTrO$(CH_2CH_2O)_6$–P(Cl)OCH_2CH_2CN and TfN$(CH_2)_6$–P(Cl)OCH_2CH_2CN were synthesized according to the literature. The duplex was cleaved from the resin with 28% aqueous NH_3 and the solution was heated at 55–60 °C for 5–6 h. Then the crude material was purified by HPLC (Nacalai tesque, Cosmosil 5C 18-MS, solvent: %B = 10→20%/20 min, 100%/30 min, B = CH_3CN, A = 0.1 M TEAA) to afford the aminolink-conjugated target duplex **3**. The synthesis of the target duplex **3** without aminolink was synthesized by the same method except that TfN$(CH_2)_6$–P(Cl)OCH_2CH_2CN was not applied at the final step.

Aminolink-conjugated target duplex **3**: MALDI-TOF mass: calcd 5397.13, found 5398.63. Target duplex **3** without amino link: MALDI-TOF mass: calcd 5217.98, found 5217.84. UV melting was measured at 260 nm with the use of the target duplex **3** (2 μM) in 0.1 M KCl buffer at pH 7.0 by raising and lowering the temperature at 1 °C/min, and melting temperature of 53 °C was obtained.

Synthesis of the magnetic beads conjugated with amino link target duplex (2)

Magnetic beads (MPG long chain alkylamine, 500 Å–5 μm, 1.2–1.8×10^8 particles/30 mg in 1.0 mL) were put

into a plastic tube, then the tube was placed on a strong magnet and the supernatant was taken out. Coupling buffer (3.0 mL, 10 mM phosphate, pH 7.5) was added to the beads, and the whole was mixed well. The beads were magnetically separated and washed with the same buffer (3.0 mL×5). A solution of 10% glutaraldehyde (3.0 mL, in the above buffer) was added to the above beads, and the mixture was gently rotated for 2 h. The solvent was removed and the beads were washed with the buffer (3.0 mL×5). A solution of the aminolink-conjugated target duplex DNA (**3**) (3 mg, 3.0 mL coupling buffer) was added to the above beads, followed by the addition of a solution of % $NaCNBH_3$ (300 μL in coupling buffer). The reaction mixture was rotated for 11.5 h at room temperature. The beads were washed with coupling buffer, followed by treatment of 1% glycine solution (in 3 mL, coupling buffer) and 1% $NaCNBH_3$ solution (300 μL in the coupling buffer) for 3 h at room temperature. The beads were washed with washing buffer (10 mM phosphate, pH 7.0, 1.0 mM NaCl) (3.0 mL×5) to give the target-conjugated magnetic beads (**2**), which were stored in a refrigerator (4 °C). A part of the DNA-bound magnetic beads (3.0 mg) was treated with venom phosphodiesterase (VPDE) and bacterial alkaline phosphatase (BAP) in a buffer for 2 h at room temperature, then the mixture was centrifuged (10,000 rpm) to remove the magnetic beads. The supernatant was analyzed by HPLC (Nacalai tesque, Cosmosil packed column, 4.6×250 mm, 5C 18-MS, solvent: %B = 5→20%/20 min, 100%/30 min, flow rate: 1 mL/min, $B = CH_3CN$, A = 0.05 M NH_4OOCH, UV = 260 nm). The HPLC chromatogram, is shown in Figure 4, clearly showing the four base peaks in an almost expected ratio.

Synthesis of the pentapeptide library beads

6-(*N*-*t*-Buthytoxycarbonylamino)caproic acid. Di-*t*-butoxycarbonate (11.8 g, 0.057 mol,) and NaOH (2.2 g, 0.055 mol) was added into a solution of 6-aminocaproic acid (6.56 g, 0.05 mol) in *t*-butanol (40 mL), and the mixture was stirred for 5 h at room temperature. The reaction mixture was quenched with water (55 mL), and the whole was extracted with *n*-hexane (300 mL×2). The aqueous layer was acidified with 10% HCl at pH 2 at 0 °C, and salted out with NaCl, then extracted with EtOAc (300 mL×2). The combined organic layers were washed with brine (300 mL×2) and dried over Mg_2SO_4, filtered and evaporated. The solvent was evaporated to give a crude oil, which was crystallized from *n*-hexane to give colorless fine needles (9.68 g, 88%). mp: 32.5–33.5 °C. IR (cm^{-1}): 3340, 3400–2400, 1710, 1525, ^1H NMR (270 MHz, $CDC1_3$/TMS): 10.69 (1H, bs), 4.61 (IH, bs), 3.10–3.08 (2H, m), 2.32 (2H, t, $J = 7.4$ Hz), 1.63 (2H, q, $J = 7.6$ Hz), 1.53–1.29 (4H, m), 1.42 (9H, s), FABMS (*m*/*z*): 463 $(2M + H)^+$, 232 $(M + H)^+$.

Incorporation of 6-(*N*-*t*-buthyloxycarbonylamino)caproic acid onto the Merrifield resin. 6-(*N*-*t*-Buthyloxycarbonylamino)caproic acid (4.6 g, 21 mmol) was added into a solution of KO*t*Bu (3.5 g, 0.012 mol) in dry DMSO (50 mL), followed by addition of the Merrifield resin (chloride form, 5 g, 4.8 mmol). The mixture was heated at 80 °C for 2 h with occasional shaking. The beads were collected on a glass filter and washed with DMSO (10 mL×3), CH_2Cl_2 (20 mL×5) and dried under vacuum to give the reacted beads (6.2 g). The beads (5.36 g) were treated with CF_3COOH/CH_2Cl_2 solution (4–6 mL) for 30 min at room temperature, and collected on a glass filter, washed with CH_2Cl_2 (50 mL×2) and dried under vacuum to give the beads (5.14 g). The above beads (10.0 g, 2.3 mmol) were suspended in *N*-methylpyrrolidone (20 mL), followed by addition of 20 mL of 0.5 M DMF solution (10.0 mmol) of HBTU (2-(1-H-benzotriazol-l-yl-)-1,1,3,3,-tetramethyluronium hexafuluorophosphate) and HOBt (1-hydroxy-benzotriazol hydrate) and diisopropylethylamine (1.8 mL, 10 mmol). Into the above mixture was added 6-(*t*-butoxycarbonylamino)caproic acid (1.5 g, 6.9 mmol), and the mixture was stirred for 1 h at room temperature. The beads were collected on a glass filter, and washed successively with *N*-methylpyrrolidone (50 mL×2) and CH_2Cl_2 (50 mL×2), and dried under vacuum to give the beads with two units of 6-aminocaproic acid (10.5 g). The above beads (8.52 g, 5.03 mmol) were treated with 50% TFA-CH_2Cl_2 (60 mL) for 1 h at room temperature. The beads were collected on a glass filter, and washed with CH_2Cl_2 (10 mL×5) and dried under vacuum to give the beads with free amino groups (8.4 g).

Elongation of peptide chain by the pool and split method. The above beads were divided into 20 vessels, and the peptide chain elongation was done as follows. The mixture of the above beads (0.55 g), Fmoc-amino acid (1.0 mmol), diisopropylethylamine (0.35 mL, 2.02 mmol) and 3.5 mL of 0.5 M DMF solution of HBTU-HOBT (1.75 mmol) in *N*-methylpyrrolidone (3.5 mL) were shaken gently for 0.5 h at room temperature. The beads were collected on a glass filter and successively washed with *N*-methylpyrrolidone (5 mL×3), methanol (5 mL×3), CH_2Cl_2 (5 mL×3). This washing procedure was repeated twice and dried under vacuum. At this stage, the reaction completion was monitored by the picrate method. When the reaction was not completed, the same coupling procedure was repeated. The peptide-elongated beads were treated with 20% piperidine in *N*-methylpyrrolidone for 2 h at room temperature, and were collected on a glass filter. The beads were washed successively with *N*-methylpyrrolidone (2 mL×3) and methanol (2 mL×3). This washing procedure was repeated twice. The above peptide elongation was done separately with using 20 Fmoc-protected amino acid, and the obtained beads were combined and mixed well, then divided into 20 vessels again according to Lam's protocol.[21] Then the same elongation procedure was repeated to give library beads containing all the pentapeptide sequences constructed based on the one-bead-one-sequence concept.

Selection procedure of the library beads with the target conjugated magnetic beads. A suspension of the library beads (200 mg) was gently rotated for 3 h in a buffer containing 100 mM NaCl and 5 mM Na_2HPO_4 at pH 7.0 at room temperature. The DNA-conjugated magnetic beads (5.4 mg in 0.2 mL) were added to the above suspension, and the mixture was slowly rotated for 3 h

at room temperature. A strong magnet was placed outside the wall of the test tube. The interacting bead–bead complexes adhered to the magnetic position on the inside wall of the tube, and were collected. A single bead–bead complex was separated from the mixture by a fine glass needle under a microscope, and was directly loaded to a peptide sequencer. The whole operation is schematically shown in Figure 2.

Synthesis of pentapeptides. The peptides used in this study were synthesized by using HMP resin (1.1 mmol/g) by a standard *N*-Fmoc chemistry. At first, two units of 6-aminocaproic acid were introduced. Under argon, the mixture of the HMP resin (3.0 g, 3.3 mmol), 9-fluorenylmethyloxycarbonyl-6-aminocaproic acid (5.63 g, 16.5 mmol), DCC (*N*,*N*′-dicyclohexylcarbodiimide) (3.40 g, 16.5 mmol) and DMAP (4-dimethylaminopyridine) (0.40 g, 3.3 mmol) in *N*-methylpyrrolidone (30.0 mL) was shaken gently for 2.5 h at room temperature. The beads were collected on a glass filter and successively washed with *N*-methylpyrrolidone (10.0 mL×4), methanol (10.0 mL×4), CH_2Cl_2 (10.0 mL×4) and dried under vacuum. The above beads were treated with 20% piperidine in DMF (30.0 mL) for 80 min at room temperature and were collected on a glass filter. The same procedure was done again to give the beads conjugated with two units of 6-aminocaproic acid. The pentapeptides used in this study were synthesized using the above beads by standard Fmoc chemistry as described for the synthesis of library beads. The cleavage of the synthesized peptide was done with 50% TFA in CH_2Cl_2 (4.0 mL) in the presence of 3% triisobutylsilane and 2% H_2O for 1 h at room temperature. The reaction mixture was filtered, and the filtrate was diluted with cold ether (100.0 mL). White precipitates were collected on a glass filter, washed with ether, then dissolved in TFA (1–1.5 mL). TFA solution was evaporated to dryness and the crude peptide was purified by HPLC (Nacalai tesque, Cosmosil, 5C 18-MS, Solvent: %B = 35%, %A = 65%, flow rate = 10 mL min, UV = 254 nm, B = 0.05% TFA in CH_3CN, A = 0.05% TFA in H_2O). Freeze-drying of the solvents afforded the desired peptides as a white powder whose structures were confirmed by ^1H NMR (500 MHz) and FAB mass spectrometry.

Binding experiments. Fluorescence spectra were recorded on a F-2000 or a Jasco FP-750 fluorescence spectrophotometer at room temperature using a solution in 0.01 M SHE buffer (9.4 mM NaCl, 2 mM Hepes, and 10 μM EDTA, pH 5.0). DNA was dissolved in the measurement buffer and was annealed from 80 to 10 °C during 70 min. Stock solutions of ethidium bromide and peptide were freshly prepared immediately before use. Emission spectra of ethidium bromide were recorded between 550 and 650 nm at excitation wavelength of 546 nm, and changes of intensity at 595 nm were analyzed by the least squares method to give the binding constant. Job plots were obtained by measuring emission intensity of 10 samples, including 5 μM total concentration of ethidium bromide and DNA in a different ratio, and an example result is shown in Figure 6. Fluorescence quenching of FQWII was done by adding a solution of DNA by portions into a solution of FQWII (1.0 μM in 10 mM SHE buffer). Changes in emission intensity at 350 nm with excitation wavelength of 296 nm were analyzed. In the experiments of ethidium-displacement assay, the changes in fluorescence intensity at 595 nm with excitation at 546 nm were measured by adding a stock solution of the peptide into a solution of ethidium bromide (1.0 μM) and DNA (1.0 μM) in 10 mM SHE buffer at room temperature. The C_{50} value represents the concentration needed for 50% decrease of fluorescence intensity (Table 2).

Acknowledgments

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (C09672146 to S.S.). Scholarship to M. R. A. from the Ministry of Education, Science, Sports and Culture, Japan is also appreciated.

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