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別紙4

研究成果の刊行に関する一覧表レイアウト（参考）

書籍　　　該当なし

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Effects of DNA Binding of the Zinc Finger and Linkers for Domain Fusion on the Catalytic Activity of Sequence-Specific Chimeric Recombinases Determined by a Facile Fluorescent System

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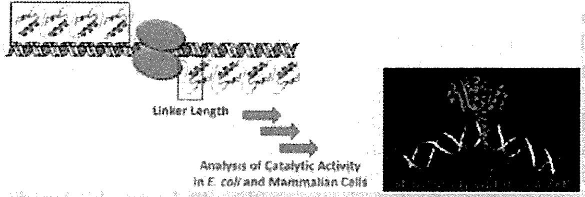
Ⓢ *Supporting Information*



ABSTRACT: Artificial zinc finger proteins (ZFPs) consist of Cys_2-His_2-type modules composed of ~30 amino acids with a $\beta\beta\alpha$ structure that coordinates a zinc ion. ZFPs that recognize specific DNA target sequences can substitute for the binding domains of enzymes that act on DNA to create designer enzymes with programmable sequence specificity. The most studied of these engineered enzymes are zinc finger nucleases (ZFNs). ZFNs have been widely used to model organisms and are currently in human clinical trials with an aim of therapeutic gene editing. Difficulties with ZFNs arise from unpredictable mutations caused by nonhomologous end joining and off-target DNA cleavage and mutagenesis. A more recent strategy that aims to address the shortcomings of ZFNs involves zinc finger recombinases (ZFRs). A thorough understanding of ZFRs and methods for their modification promises powerful new tools for gene manipulation in model organisms as well as in gene therapy. In an effort to design efficient and specific ZFRs, the effects of the DNA binding affinity of the zinc finger domains and the linker sequence between ZFPs and recombinase catalytic domains have been assessed. A plasmid system containing ZFR target sites was constructed for evaluation of catalytic activities of ZFRs with variable linker lengths and numbers of zinc finger modules. Recombination efficiencies were evaluated by restriction enzyme analysis of isolated plasmids after reaction in *Escherichia coli* and changes in EGFP fluorescence in mammalian cells. The results provide information relevant to the design of ZFRs that will be useful for sequence-specific genome modification.

Artificial zinc finger proteins (ZFPs) can be used to engineer DNA binding domains with high specificity for desired target sequences, and ZFPs are a promising technology for gene therapy.[1−6] Modular assembly of ZFPs can create a DNA binding domain that targets virtually any sequence in the human genome.[3−5] By linking ZFPs to the catalytic domains of DNA-modifying enzymes, novel enzymes, including nucleases,[6] recombinases,[7−12] and methylases,[13−20] have been fabricated. These enzymes are endowed with programmable DNA binding specificity provided by the zinc finger protein fusion. Relevant to our development of ZFRs, recombinase enzymes from the serine recombinase family have been well studied.[21] In comparison with members of the tyrosine recombinase family such as Cre and Flp recombinases, the serine recombinases, including Tn3 and $\gamma\delta$ resolvases, Hin invertase, and Gin invertase, have DNA binding domains that are structurally independent of the catalytic domain. The structures of the catalytic domains and the sequences required for catalytic activity are highly conserved in these recombinases.[22] Tn3 and $\gamma\delta$ are among the best-characterized site-specific recombinase enzymes in the serine recombinase family. Only 35 amino acid residues differ between the $\gamma\delta$ and Tn3 resolvases, and their structures and functions are similar.[23] Negatively supercoiled DNA is a prerequisite for substrate recombination with native serine recombinase enzymes.[21] Although it is known that native serine recombinases require accessory proteins binding to sites I−III, activating mutants that require only the 28 bp of site I for successful recombination have been isolated.[7] In these hyperactivated enzymes, a DNA substrate in the form of negatively supercoiled DNA is not required for activity, and this allows application of activated catalytic domains with ZFPs to create zinc finger recombinases (ZFR). It has been suggested that reactions with serine recombinases proceed in three

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steps: (i) formation of a dimer binding to the two forms of site I on the DNA, (ii) formation of a tetramer between the forms of site I, and (iii) strand exchange.[24,25] After the strand exchange reaction, the sequences between target sites are excised and the strands ligated (Figure 1).

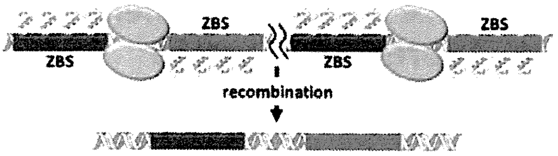

Figure 1. Schematic illustration of the ZFR reaction at a target site. The green and red boxes represent zinc finger binding sites (ZBSs). The yellow spheres represent catalytic domains of Tn3 resolvase.

ZFRs based on catalytic domain variants of Tn3, Gin, and Hin fused to artificial ZFPs have been shown to catalyze site-specific recombination in *Escherichia coli*[7−11] and mammalian cells.[8,9,11,12] ZFRs have also been shown to catalyze high-fidelity site-specific integration in mammalian cells.[9,11,12] While directed evolution of recombinase catalytic domains has proven to be essential for developing ZFR enzymes that function in mammalian cells, other aspects of ZFR design have not been thoroughly studied. In this report, we have synthesized ZFR mutants with variable numbers of zinc fingers and studied the role of peptide linkers that connect the Tn3 resolvase catalytic domain with the ZFP DNA binding domain. These effects are not readily addressed using molecular evolution strategies. For facile evaluation of recombination reactions in mammalian cells, a system that allows evaluation within 48 h was developed utilizing DsRed expression as a marker of transfection efficiency and EGFP expression as a marker of recombination efficiency. The results obtained revealed the optimal structures of the ZFRs, and the recombination efficiency results for linker mutants were verified by modeling studies.

■ EXPERIMENTAL PROCEDURES

Construction of ZFP Genes. ZFP genes were constructed as described previously.[26,27] Briefly, plasmid pc3XB encoding ZFPs purchased from Addgene (http://www.addgene.org) was repeatedly ligated. The zinc finger gene that was obtained was inserted into pMAL-p4x as an *Xba*I−*Bam*HI fragment for protein expression. A minor change was made to the multiple cloning site of pMAL-p4x (Figure S1 of the Supporting Information).

Target Enzyme-Linked Immunosorbent Assays (ELISAs). ELISA wells of 96-well plates were coated by incubation with 25 μL of 8 ng/mL streptavidin in PBS for 1 h at 37 °C. The plates were washed twice with dH_2O, and 25 μL of 5′-biotinylated hairpin oligonucleotide target in zinc buffer A (ZBA) [10 mM Tris-HCl (pH 7.5), 90 mM KCl, 1 mM $MgCl_2$, and 90 μM $ZnCl_2$] was added. After incubation for 1 h at 37 °C, plates were washed twice with dH_2O. Blocking solution (ZBA with 3% BSA, 175 μL) was added, and incubation continued for 1 h at 37 °C. The blocking solution was then removed; 25 μL of purified protein in ZBA was added, and 2-fold serial dilutions were performed into 1% BSA, 5 mM DTT, and 10 ng/μL salmon sperm DNA in ZBA. After incubation for 1 h at room temperature, the plates were washed 10 times with dH_2O and the monoclonal anti-MBP antibody (Sigma-Aldrich, 1:1000 dilution by ZBA with 1% BSA, 25 μL) was added. After incubation for 30 min at room temperature, the plates were washed 10 times with dH_2O and a diluted secondary anti-mouse IgG AP conjugate (Sigma-Aldrich, 1:1000 dilution by ZBA with 1% BSA, 25 μL) was added. After incubation for 30 min at room temperature, plates were washed 10 times with dH_2O. The alkaline phosphatase reaction was performed with *p*-nitrophenylphosphate for 30 min, and the absorbance at 405 nm was read with a microplate reader. The data were collected and plotted. The data were fit to the equation $y = 1/(1 + K_d/x)$, where y is the proportion of bound MBP−ZFP fusion protein to maximal binding derived from the absorbance at 405 nm and x is the concentration of the MBP−ZFP fusion protein. The K_d values are averages of three or more independent experiments, and standard errors of the mean (SEM) are shown.

Construction of ZFR Substrates. Each substrate plasmid contained a recombination cassette composed of two ZFR recombination sites flanking an EGFP gene as a stuffer sequence. Cassettes were assembled by amplifying the EGFP gene with primers encoding the ZFR site. The polymerase chain reaction (PCR) product was cloned into pAra-OP.[20] ZFP genes were amplified by PCR from plasmid pc3XB and inserted into the plasmid as *Eco*RI−*Sac*I fragments. Plasmids that contained ZFR with Gly-Ser linkers were mutated at the *Bst*BI site before insertion of the catalytic domain.

Construction of ZFR Genes. The DNA fragment of the Tn3 resolvase catalytic domain was amplified from pWL625 (ATCC accession number 31787) utilizing 5′-GAGGAGGAATTCATGCGACTTTTTGGTTACGCT-3′ and 5′-GAGGAGAAGCTTTCACGAGGCCCTTTCGTCTT-3′ as primers. The fragment was inserted into pBluescriptSK(−) as an *Eco*RI−*Hin*dIII fragment. Tn3-activating mutations (R2A, E56K, G101S, D102Y, M103I, and Q105L) were introduced into the Tn3 encoding gene. Linker sequences were amplified via PCR with the Tn3 fragment by primers that included the linker sequence. Tn3 fragments with different linkers were digested with *Eco*RI and *Bgl*II and ligated into similarly digested pAra-OP with the EGFP and ZFR sites. Tn3 fragments with various Gly-Ser linkers were also digested with *Eco*RI and *Bst*BI and then ligated. The plasmids were maintained with chloramphenicol.

Assay of Recombination of Plasmids in *E. coli*. The plasmid with a ZFR gene downstream from the arabinose promoter and the substrate sequences were introduced into *E. coli* by electroporation. After incubation for 14 h at 37 °C on an LB-agar plate, colonies were picked up and grown for 14 h at 37 °C in LB medium. Purified plasmids were digested with *Eco*RI for 1 h at 37 °C. After electrophoresis on a 0.8% agarose gel, the fragment intensity was estimated with ImageJ (Figure S2 of the Supporting Information).

Recombination Reaction of ZFR in Mammalian Cells. The EGFP gene, flanked by recombination sites, was inserted between *Nhe*I and *Kpn*I in pcDNA5/FRT (Life Technologies). A double-stranded oligonucleotide encoding the upstream target site was inserted into the *Mlu*I site, and the other oligonucleotide for the downstream target site was inserted into *Kpn*I and *Bam*HI sites. Cotransfection of the substrate plasmid and Flp expression plasmid (pOG44, Life Technologies) allowed site-specific integration into the single FLP recombinase target (FRT) site present in the Flp-In-CHO cell line (Life Technologies). Colony-acquired hygromycin resistance was characterized by fluorescently activated cell sorting (FACS) and genomic PCR. The sequence of the target site was confirmed. Cells were maintained in Ham's F-12 containing 10% (v/v)

Table 1. DNA Binding Affinities of ZFPs

	two fingers	three fingers	four fingers	five fingers	six fingers
K_d (nM)[a]	160±20	23.6±3.6	12.8±1.1	15.4±1.4	12.9±1.4
R^2	0.90	0.87	0.94	0.94	0.94

[a] The values are averages of three or more independent experiments.

FBS and antibiotics (Wako Chemicals). The DsRed expression vector was constructed as follows; a DsRed-monomer sequence was ligated into pIRES2-EGFP (Clontech) to substitute for EGFP, and a Tn3-ZFP-NLS fragment was inserted between *Nhe*I and *Eco*RI in pIRES2-DsRed. On the following day, after 2 × 10^5 cells had been seeded, the ZFR expression vector was transfected into cells using Lipofectamine LTX Reagent and PLUS Reagent (Life Technologies). After being transfected for 48 h, cells were collected and analyzed by flow cytometry.

Molecular Modeling of the Linker Variants of ZFR. Computer models were generated using Discovery Studio (Accelrys Inc.). The crystal structure of the γδ resolvase–DNA complex [Protein Data Bank (PDB) entry 1GDT][22] was manually mutated in the protein and DNA to match the molecules used in this study. The first zinc finger module, obtained from a zinc finger–DNA complex (PDB entry 1MEY),[28] was placed on the resolvase–DNA complex by superimposing the phosphate backbone atoms of corresponding DNA residues. Appropriate linker atoms were then added and optimized by simulated annealing and energy minimization. During this optimization, the atoms in the resolvase, zinc fingers, and DNA were fixed, allowing only linker atoms to move.

■ RESULTS

Construction of Zinc Fingers and DNA Binding Analyses. The 18 bp target sequence of the zinc finger protein utilized in this study was 5′-CTGCATGCACTGGATGCA-3′.

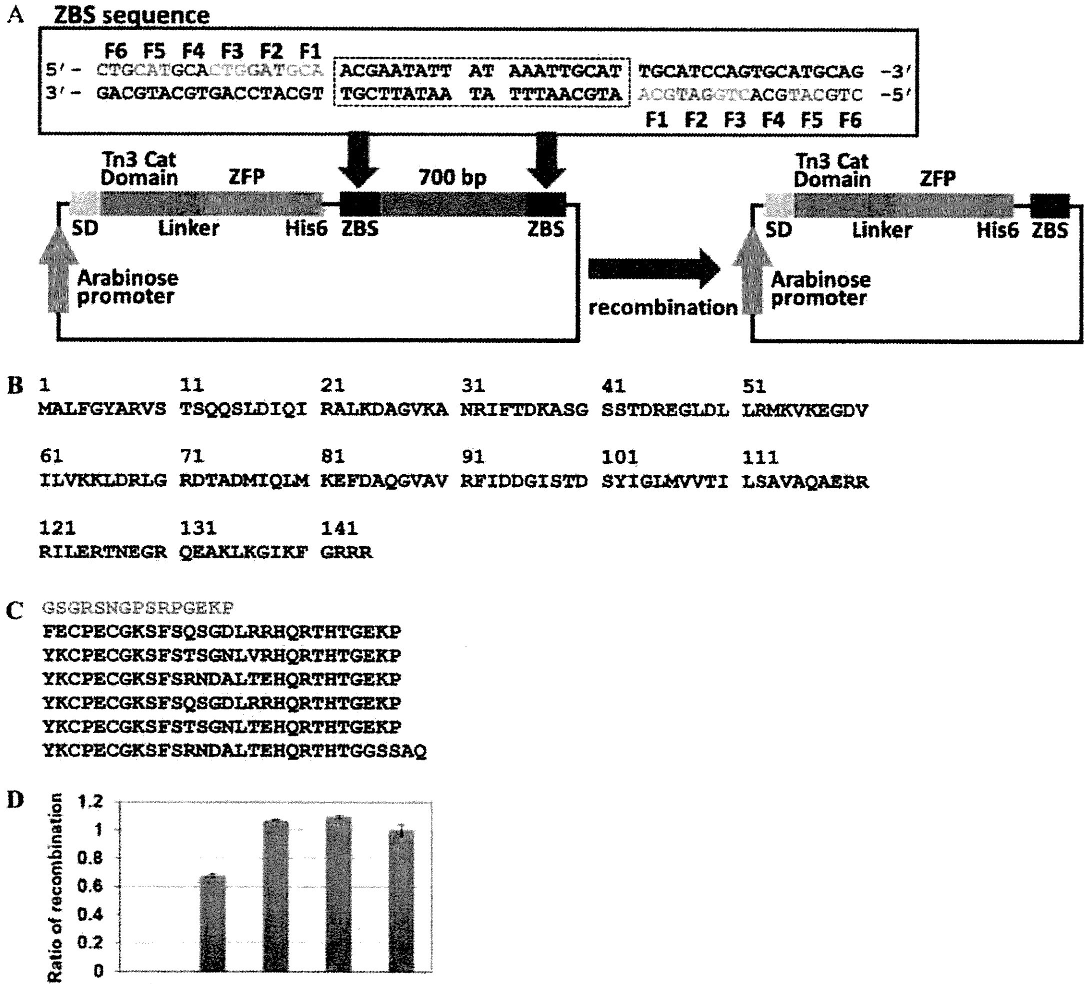

Figure 2. (A) Schematic of recombination at zinc finger binding sites (ZBSs). Recombination results in smaller plasmids. ZBS sequences are shown in the box. SD represents the Shine-Dalgarno sequence. (B) Amino acid sequences of the hyperactivated Tn3 catalytic domain. (C) Amino acid sequences of the linker (red) and six-zinc finger domain utilized for the analysis in *E. coli*. (D) Recombination efficiency depends on the number of fingers in ZFR. Columns 1–5 show the recombination efficiencies of two- through six-finger modules. The ratios are relative to the efficiency of the six-finger module. The error bars show the SEM of three or more independent experimental results.

A

```
Clone 1.         R RSNGPSRPGEKP
Clone 2. IKF     R RSNGPSRPGEKP
Clone 3. IKF GRRR
Clone 4. IKF GRRR EKP
Clone 5. IKF GRRR RPGEKP
Clone 6. IKF GRRR GPSRPGEKP
Clone 7. IKF GRRR RSNGPSRPGEKP
Clone 8. IKF GRRR GSGRSNGPSRPGEKP
Clone 9. IKF GRRR GSGGGSRSNGPSRPGEKP
Clone 10.IKF GRRR GSGGGSGGSRSNGPSRPGEKP
Clone 11.IKF GRRR GSGGGSGGSGGSRSNGPSRPGEKP
Clone 12.IKF GRRR GSGGGSGGSGGSGGSRSNGPSRPGEKP
Clone 13.IKF GRRR GSGGGSGGSGGSGGSGGSRSNGPSRPGEKP
Clone 14.IKF GRRR GSG
Clone 15.IKF GRRR GSGGGS
Clone 16.IKF GRRR GSGGGSGGS
Clone 17.IKF GRRR GSGGGSGGSGGS
Clone 18.IKF GRRR GSGGGSGGSGGSGGS
Clone 19.IKF GRRR GSGGGSGGSGGSGGSGGS
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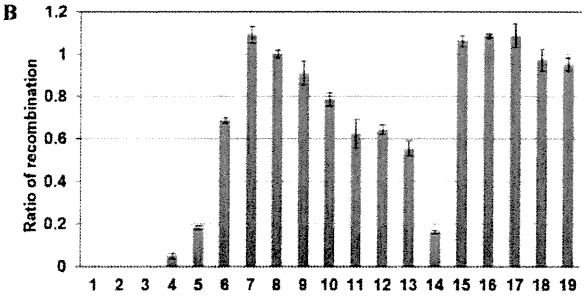


Figure 3. (A) Amino acid sequences of linkers of clones. All linkers were tested in the context of six-finger binding domains. (B) Results of recombination efficiency of clones with different linker sequences. The numbers of columns correspond to the clone numbers as described in panel A. The ratios are relative to the efficiency of clone 8. The error bars show the SEM of three or more independent experimental results.

Zinc fingers were constructed on the basis of a modular assembly strategy described by Barbas and co-workers.[27,29−32] Two- to six-finger proteins were constructed to obtain DNA binding domains with different affinities. Proteins were expressed as maltose binding protein fusions and purified with an MBPTrap column (GE Healthcare). The purity of the proteins was determined to be >90%. The DNA binding affinities were evaluated by an ELISA with the biotinylated hairpin oligonucleotide as a target.[10] The binding constants (K_d) of the two-, three-, four-, five-, and six-finger modules, listed in Table 1, were found to be 160, 23.6, 12.8, 15.4, and 12.9 nM, respectively. These results indicate that in the two-, three-, and four-finger modules, the DNA binding affinity increased with finger number but the binding affinities of ZFPs with four, five, and six fingers were similar.

Construction of ZFR Chimeric Proteins and Recombination Analysis in *E. coli*. The target DNA sequence of ZFR is shown in Figure 2A. The target site consists of a 20 bp spacer sequence flanked by 18 bp zinc finger binding sites. The spacer region was previously shown to be a Z+4 site in the target spacer of Z-resolvase.[7] For the evaluation of recombination in *E. coli*, a plasmid-based recombination system was constructed. The coding sequence of ZFRs was inserted into the plasmid containing a 700 bp stuffer sequence flanked with target sequences. In the recombination mediated by the expressed ZFRs, the stuffer sequence is excised to produce a smaller plasmid (Figure 2A). The amino acid sequences of the hyperactivated Tn3 catalytic domain, the linker between the domains, and the zinc finger domain are shown in panels B and C of Figure 2. The recombination efficiency was evaluated by a restriction enzyme assay. Plasmid purified from *E. coli* was digested by *Eco*RI, which is a single cutter of the plasmid. The linear plasmid was analyzed on an 0.8% agarose gel, and the fractions of the longer (nonrecombinant) and shorter (recombinant) plasmids were evaluated (Figure S2 of the Supporting Information). ZFR variants with different numbers of fingers were evaluated in this recombination system, and recombination ratios increased with increasing numbers of fingers from two to four fingers. The values of recombination efficiencies for ZFRs with four to six fingers were similar, reflecting the DNA binding affinities (Figure 2D). The production of recombinant sequence was confirmed by DNA sequencing analysis (Figure S3 of the Supporting Information).

In the next study, the reactions of ZFR variants with different linker lengths in the context of the six-finger module were tested (Figure 3B). In this experiment, 19 constructs were prepared. The variants were categorized into three groups depending on lengths and the compositions of linker sequences. The first group variants have short linkers with deletions within the catalytic

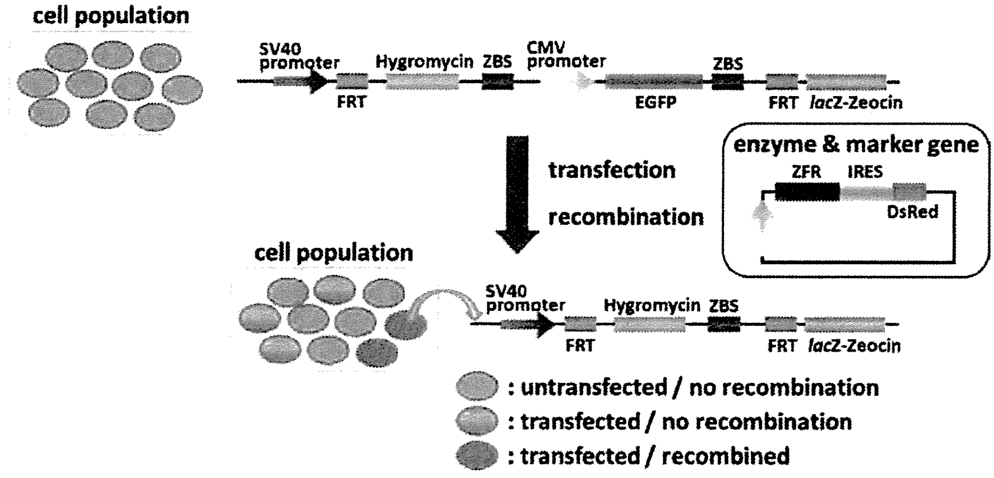


Figure 4. Recombination system constructed utilizing Flp-In-CHO-K1 cells.

domain of Tn3 resolvase (clones 1 and 2). The second group of variants has semirigid linkers (clones 3−13). The third group has flexible linker sequences composed of Gly-Ser sequences (clones 14−19). In the clones of the third group, the first two amino acids of the zinc finger domain, Tyr and Lys, are substituted with Phe and Glu, respectively. The recombination efficiencies were determined in the *E. coli*-based assay (Figure 3B). The results indicate two important phenomena. (1) The variant with a 12-amino acid linker was the most efficient (clone 7, Figure 3A), suggesting that there is an optimal linker length. (2) The variants with linkers composed of only Gly-Ser sequences were most efficient (clones 15−17, Figure 3A), indicating that ZFRs with flexible linkers tended to recombine most efficiently.

ZFR-Catalyzed Recombination in Mammalian Cells. To evaluate the recombination efficiency of ZFR variants in mammalian cells, we constructed a reporter cell line from Flp-In-CHO-K1 containing a cassette that encodes EGFP driven by a CMV promoter flanked by target sites (Figure 4). As each cell contains a single copy of the reporter gene, the recombination efficiency can be calculated from the proportion of cells with or without EGFP fluorescence. Additionally, the expression of ZFR was monitored by the expression of DsRed; this gene was placed downstream of the ZFR gene via a IRES sequence. The genes encoding ZFRs utilized in this study were amplified from a pAra plasmid shown in Figure 2A. Thus, the sequences of clones are the same as those utilized in experiments in *E. coli*.

With this reporter system, recombination efficiencies could be evaluated 48 h after transfection. Reported procedures involving retroviral-based transduction, selection, and evaluation take nearly 10 days.[8] The fluorescence intensity of cells was detected by FACS analysis (Figure S4 of the Supporting Information). The cells with recombinant genes were those that were EGFP-negative and DsRed-positive. The recombination efficiencies depended on the number of finger modules and on the linker lengths (Figure 5). As in *E. coli*, the five-finger proteins were the most efficient in recombination. The optimal linker length was six residues, which is different from that in *E. coli*. Additionally, recombination in mammalian cells was not as efficient as that in *E. coli*.

■ DISCUSSION

This study demonstrated that ZFR recombinases can be designed to specifically target sites in *E. coli* and mammalian cells and that recombination efficiency depends on the affinity of the ZFP for the DNA target and on the length of the linker between the DNA binding domain and the recombinase domain. The ZFR with five fingers had the highest recombination efficiency in both *E. coli* and CHO-K1 cells. The DNA binding affinity of this particular ZFP was saturated when the DNA binding domain had more than five fingers. The association and dissociation with DNA binding depend on the number of finger modules.[33] It is possible that the ZFR with five fingers was the most efficient recombination because the balance of association with dissociation and turnover was optimal. Guo et al. have also reported that four and five ZF domains are optimal for activity of ZFN.[34] On the basis of our data, the apparent K_d values of the four-, five-, and six-finger proteins derived from this particular ZFP were similar. The dependence on the number of finger modules was common in both *E. coli* and mammalian cells, but the recombination efficiency was lower in mammalian cells. In CHO-K1 cells, DNA is sequestered in chromatin structures. Additionally, the circular form of plasmid DNA could enhance recombination in the bacterial cells.

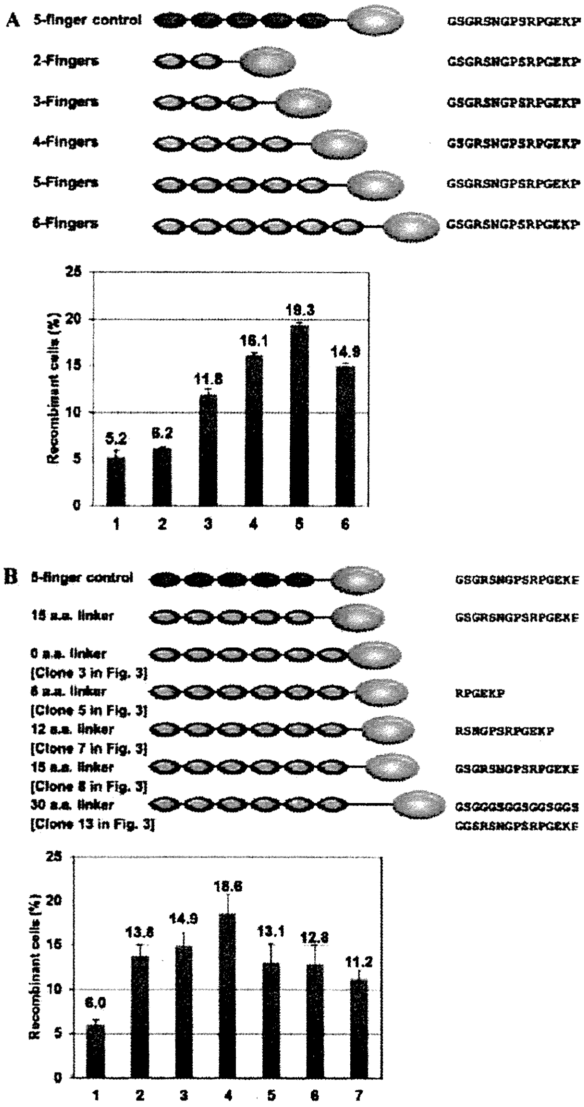
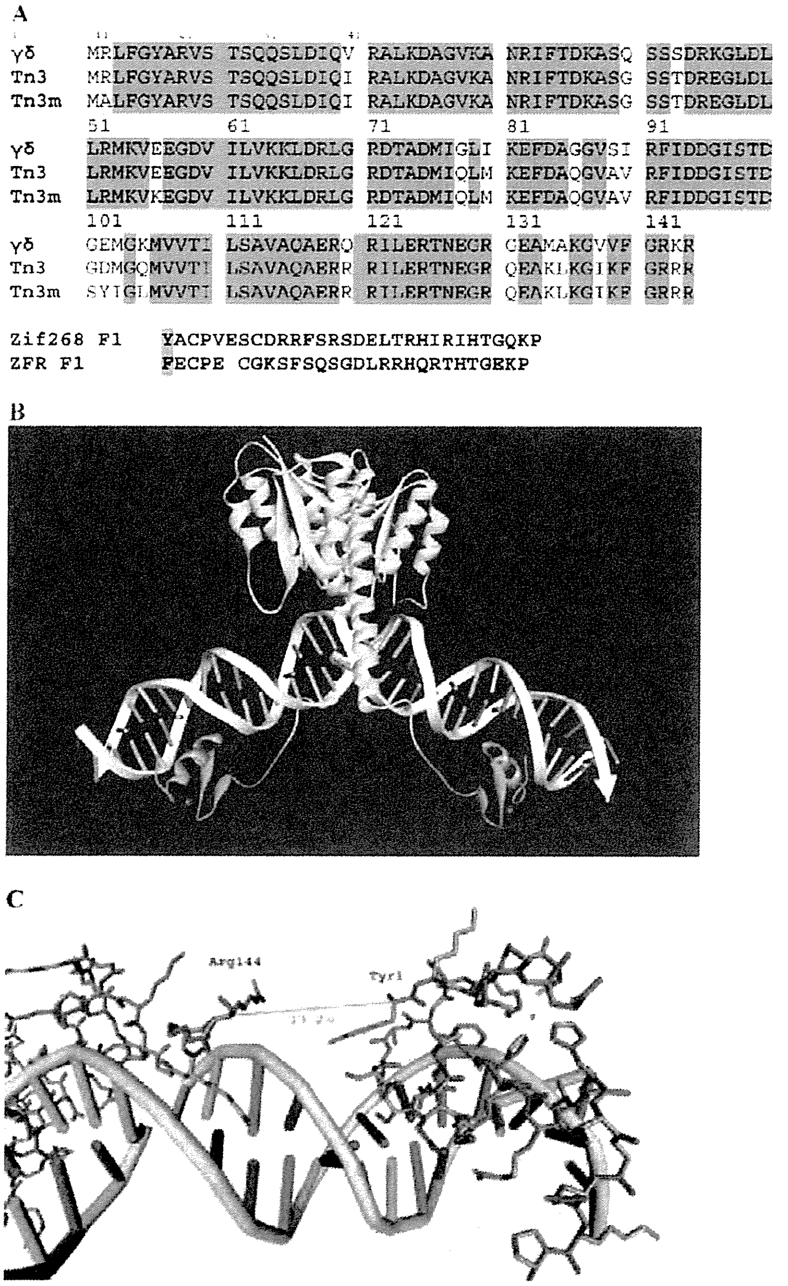


Figure 5. Recombination efficiency of ZFRs containing various numbers of fingers (A) and with various linkers (B) in mammalian cells. The top cartoons represent ZFR constructs utilized in the analyses. Green, blue, and yellow spheres represent zinc finger modules without sequence specificity, zinc finger modules with sequence specificity, and the Tn3 catalytic domain, respectively. Letters at the right of the cartoons are the linker sequences of the constructs. (A) Dependence on the number of fingers of ZFRs. The columns are as follows: column 1, five-finger control (nonspecific DNA binding); column 2, two fingers; column 3, three fingers; column 4, four fingers; column 5, five fingers; column 6, six fingers and different linker lengths. (B) Dependence on linker length. The columns are as follows: column 1, nontarget five-finger control with 15 amino acids; column 2, targeted five-finger ZFR with 15-amino acid linker; columns 3−7, targeted six-finger ZFRs with linker lengths of 0, 6, 12, 15, and 30 amino acids, respectively. The error bars show the SEM of three or more independent experimental results.

Recombination efficiency was dependent on the linker between the zinc finger domain and the recombinase domain. ZFRs with the shortest linkers had a very low efficiency of



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γδ     MRLFGYARVS TSQQSLDIQV RALKDAGVKA NRIFTDKASQ SSSDRKGLDL
Tn3    MRLFGYARVS TSQQSLDIQI RALKDAGVKA NRIFTDKASG SSTDREGLDL
Tn3m   MALFGYARVS TSQQSLDIQI RALKDAGVKA NRIFTDKASG SSTDREGLDL
       51         61         71         81         91
γδ     LRMKVEEGDV ILVKKLDRLG RDTADMIGLI KEFDAGGVSI RFIDDGISTD
Tn3    LRMKVEEGDV ILVKKLDRLG RDTADMIQLM KEFDAQGVAV RFIDDGISTD
Tn3m   LRMKVKEGDV ILVKKLDRLG RDTADMIQLM KEFDAQGVAV RFIDDGISTD
       101        111        121        131        141
γδ     GEMGKMVVTI LSAVAQAERQ RILERTNEGR QEAMAKGVVF GRKR
Tn3    GDMGQMVVTI LSAVAQAERR RILERTNEGR QEAKLKGIKF GRRR
Tn3m   SYIGLMVVTI LSAVAQAERR RILERTNEGR QEAKLKGIKF GRRR

Zif268 F1   YACPVESCDRRFSRSDELTRHIRIHTGQKP
ZFR F1      FECPE CGKSFSQSGDLRRHQRTHTGEKP
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Figure 6. Representative result of molecular modeling of the resolvase domain and the first zinc finger module separated by a six-amino acid linker sequence. (A) Sequence alignment of resolvases $\gamma\delta$ and Tn3 and the Tn3 hyperactivated mutant (Tn3m) (top), the first finger of zif268, and ZFR. Conserved residues are highlighted in red, and amino acid substitutions in the hyperactive mutant are highlighted in yellow. The N-terminal aromatic amino acids of zinc fingers are highlighted in blue. (B) The yellow ribbon indicates $\gamma\delta$ resolvase, the red ribbon the six-amino acid linker, the green ribbon the N-terminal zinc finger domain, and the gray ribbon the zinc ion. (C) Distances between Cα atoms of Arg144 and tyrosine (Tyr) at the N-terminus of zif268. The N-terminal amino acid of the zinc finger domain is phenylalanine (Phe) in ZFRs utilized in this study.

recombination in both bacterial and mammalian cells. Second, the length of linkers based on the original sequences was critical. Proteins with linkers containing 12 amino acid residues were the most efficient in recombination. In the Gly-Ser linker variants, the recombination efficiency reached a maximum at six amino acids. This result indicates that both the length and the flexibility of the linker are important.

A molecular modeling study was performed in an attempt to assess the reasons for the differences in recombination efficiency among the linker mutants. In the modeling of the ZFR complex with target DNA, the linker length of six amino acids was optimal for the DNA binding of ZFR when the linker sequence was flexible (Figure 6A). When the domains were modeled bound to the target sequence, the distance between the Cα atom of Arg144 in the $\gamma\delta$ resolvase (Figure S5 of the Supporting Information) and that of Tyr at the N-terminus of the zinc finger domain is ~13.2 Å (Figure 6B,C). In polypeptides in the extended conformation, the distance between Cα atoms of sequential amino acids is 3.8 Å. Thus, a linker consisting of three amino acids (clone 4 or clone 14) should allow the protein to bind to both DNA regions, although these ZFRs had very low recombination efficiencies. In the complex with DNA, the amino groups at positions 145 and 146 of the main chain in $\gamma\delta$ resolvase interact with the phosphate backbone of DNA and amino acids of these positions are involved in the folding of the catalytic domain (Figure S5 of the Supporting Information). In the case of clone 4, the Lys-Pro residues at the C-terminus of linker residues are involved in the folding of the zinc finger domain. Thus, these amino acids are considered to be members of both domains, not of the linker sequences. With this reasoning, the six and nine amino acids in the linkers for clones 4 and 5, respectively, are shorter than the theoretically optimal length. Moreover, in the sequences of the six- and nine-amino acid linkers, the amino acid at position 146 is Pro, which could disrupt the interaction with DNA phosphate, thus lowering the recombination efficiency. Consistent with these estimations, the Gly-Ser linker with six or nine amino acids (clones 15 and 16, respectively) showed the best recombination ratio. This evidence indicates that the residues at the C-terminus of the catalytic domain and the N-terminus of the zinc finger domain are involved in domain folding because Lys-Pro residues at the N-terminus of the zinc finger domains are not included in these clones. Variants around this optimal linker length, especially those with 12 and 15 amino acids, had similar recombination efficiencies. These results show that the flexibility of the linker is not necessary when the linker length is optimal. In mammalian cells, the variant with a linker of six amino acids (clone 5) showed the best recombination and the zero-amino acid linker (clone 3) showed better recombination than the variants with longer linkers of more than 12 amino acids. The reason for this effect is unclear, but it could be due to differences in the structures of target sites on the plasmid DNA compared to the genomic DNA. Additionally, the distances between the binding sites in these systems are different. In the genomic target, the binding sites are separated by sequences of more than 2500 bp.

In this study, a newly developed recombination system allowed measurement of recombination efficiencies of ZFRs in *E. coli* and in mammalian cells. In mammalian cells, recombination with genomic targets was evaluated within 48 h of the transient expression of recombinases. Artificial enzymes such as ZFN and ZFR have been studied mainly by using viral vector systems to deliver their genes into mammalian genomes. In a report describing utilization of the retrovirus vectors for gene delivery, the recombination efficiency was as high as ~18%.[8] In our study, we also observed up to 18% recombination in cells. This system could be utilized in future studies to evaluate function of ZFRs on specific targets.

■ ASSOCIATED CONTENT

S Supporting Information

Details of subcloning, experimental results of plasmid digestion and sequencing, results of FACS analyses, and a description of key interactions in $\gamma\delta$ resolvase. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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A Synthetic C34 Trimer of HIV-1 gp41 Shows Significant Increase in Inhibition Potency

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The development of new anti-HIV-1 drugs such as inhibitors of protease and integrase has been contributed to highly active anti-retroviral therapy (HAART) for the treatment of AIDS.[1] The entry of human immunodeficiency virus type 1 (HIV-1) into target cells is mediated by its envelope glycoprotein (Env), a type I transmembrane protein that consists of surface subunit gp120 and noncovalently associated transmembrane subunit gp41.[2] Sequential binding of HIV-1 gp120 to its cell receptor CD4 and a co-receptor (CCR5 or CXCR4) can trigger a series of conformational rearrangements in gp41 to mediate fusion between viral and cellular membranes.[3–5] The protein gp41 is hidden beneath gp120, and its ectodomain contains helical N- and C-terminal leucine/isoleucine heptad repeat domains, N-HR and C-HR. Particular regions of N-HR and C-HR are involved in membrane fusion, and 36-mer and 34-mer peptides, which are derived from N-HR and C-HR, have been designated as the N-terminal helix (N36) and C-terminal helix (C34), respectively. In the membrane fusion of HIV-1, these helices assemble to form a six-helical bundle (6-HB) consisting of a central parallel trimer of N36 surrounded by C34 in an antiparallel hairpin fashion. Synthetic peptides derived from these helices have potent antiviral activity against both laboratory-adapted strains and primary isolates of HIV-1.[6–9] They inhibit the membrane fusion stage of HIV-1 infection in a dominant-negative manner by binding to the counterpart regions of gp41 (N-HR or C-HR), blocking formation of the viral gp41 core.

Several potent anti-HIV-1 peptides based on the C-HR region have been discovered,[7,8] and T20 was subsequently developed as the clinical anti-HIV-1 drug enfuvirtide (Roche/Trimeris).[8,10–13] It is a 36-mer peptide derived from the gp41 C-HR sequence and can bind to the N-HR to prevent formation of the 6-HB in a dominant-negative fashion.[10] T20 therapy has brought safety, potent antiretroviral activity, and immunological benefit to patients, but its clinical application is limited by the development of resistance. The C-terminal helix C34 is also a C-HR-derived peptide, and contains the amino acid residues required for docking into the hydrophobic pocket, termed the "deep pocket", of the trimer of the N-HR region. This peptide potently inhibits HIV-1 fusion in vitro.[14] To date, several gp41 mimetics, especially those of N36 regions, which assemble these helical peptides with branched peptide linkers, have been synthesized as antigens.[15–19]

Recently, by using a novel template with C3-symmetric linkers of equal length, we synthesized a three-helix bundle mimetic that corresponds to the trimeric form of N36.[20] The antisera obtained from mice immunized by the peptide antigen showed strong recognition against the N36 trimer peptide with structural preference. At the same time, the trimer peptide was also investigated as a fusion inhibitor. However, the trimer N36 showed only a threefold increase in inhibition of HIV-1 fusion relative to the N36 monomer.[20] In terms of N36 content, the trimer and monomer have nearly the same inhibitory potency. This phenomenon is consistent with the results from other studies.[21–23] The multimerization of the functional unit, such as synthetic ligands against receptors, show synergistic binding and strong binding activity. Thus, we hypothesized that our strategy using C3-symmetric linkers in the design of trimer mimics of gp41 could be applied to the C34 peptide, which shows significant inhibition potency in the monomeric form. In the present study, we designed and synthesized a novel three-helical bundle structure of the trimeric form of C34. This equivalent mimic of the trimeric form of C34 was evaluated as a novel form of fusion inhibitor.

The C-terminal region of gp41 is known to be an assembly site involving a trimeric coiled-coil conformation. In the design of the C34-derived peptides C34REG-thioester (Figure 1 A) and C34REG (Figure 1 B), the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence (residues 628–661) to increase aqueous solubility, and for C34REG-thioester, a glycine thioester was fused to the C terminus. To form a triple helix corresponding precisely to the gp41 pre-fusion form, we designed the novel C3-symmetric template depicted in Figure 1 C. This designed template linker has three branches of equal length, a hydrophilic structure, and a ligation site for coupling with C34REG-thioester. The template was synthesized as shown in Scheme 1. This approach uses native chemical ligation for chemoselective coupling of unprotected C34REG-thioester with a three-armed cysteine scaffold to produce triC34e (Figure 2).[24,25]

Circular dichroism (CD) spectra of C34REG and triC34e are shown in Figure 3 A. The peptides were dissolved in 50 mM sodium phosphate buffer with 150 mM NaCl, pH 7.2. Both spectra display minima at ~200 nm, indicating that these peptides form random structures. We previously reported that the

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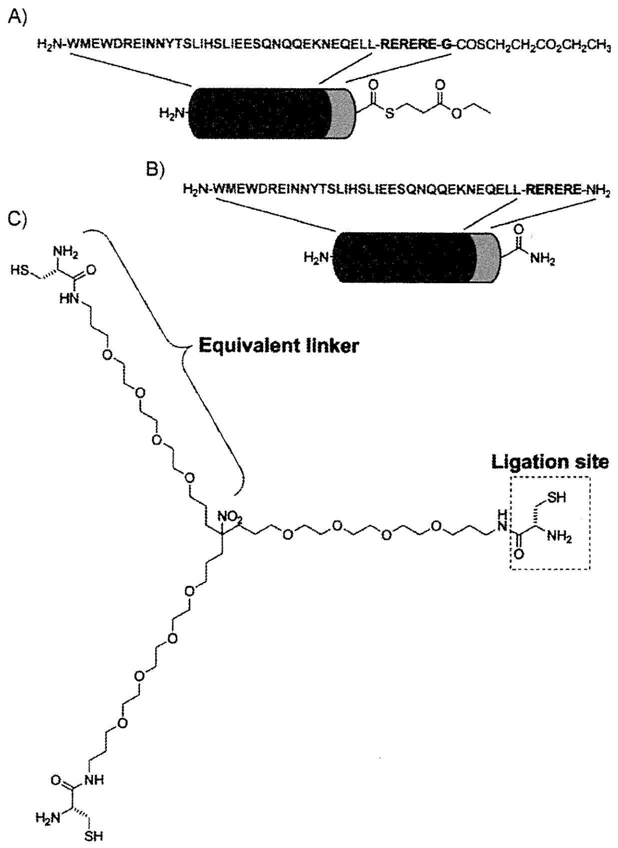


Figure 1. C34-derived peptides: A) C34REG-thioester and B) C34REG. C) The design of a C3-symmetric template.

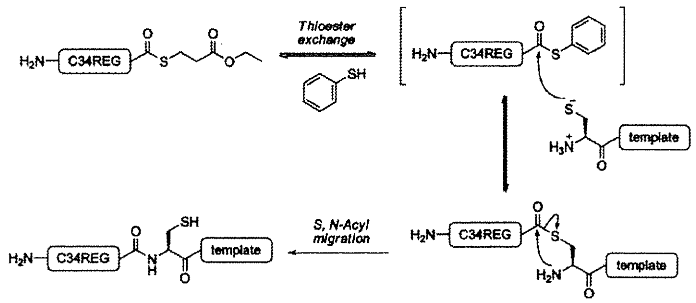
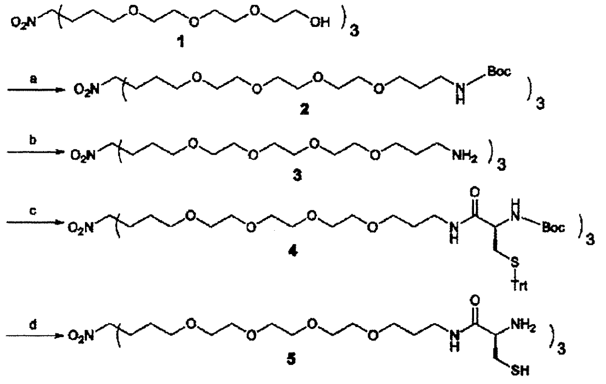


Figure 2. The native chemical ligation used for assembly of the C34REG-thioester on the template.

N36 monomer N36RE and the N36 trimer triN36e form a highly structured α helix, and that the helical content of triN36e was greater than that of N36RE.[20,26] These results suggest that in contrast to N36-derived peptides, C34-derived peptides tend to form random structures both in the monomeric and trimeric forms. To assess the interaction of triC34e with N36, CD spectra of a mixture of triC34e with an N36-derived peptide, N36RE, were measured (Figure 3B). The spectrum of the C34REG and N36RE mixture and that of the triC34e and N36RE mixture showed double minima at λ 208 and 222 nm, indicating that the peptide mixture forms an α-helical structure and that the helical content of the trimer triC34e and N36RE mixture is lower than that of the monomer C34REG and N36RE mixture. This is evidence that relative to the monomer C34REG, the trimer triC34e interacts with N36 only with difficulty, due to the assembly of three peptide strands by covalent bonds.



Scheme 1. Synthesis of the equivalently branched template **5**. *Reagents and conditions:* a) (3-bromopropyl)carbamic acid *tert*-butyl ester, NaH, THF; b) 4 M HCl/dioxane; c) Boc-Cys(Trt)-OH, EDCI·HCl, HOBt·H_2O, Et_3N, DMF; d) 90% aq. TFA.

As the trimeric C34 was proven to interact with N36 helices, the potential HIV-1 inhibitory activities of the C-terminal peptides, C34REG and triC34e, were evaluated. The C34 peptide without the solubility-increasing sequence (3×[Arg-Glu], obtained from NIAID) was used as the monomeric control.[27] All peptides showed potent inhibitory activity in the viral fusion assay (Table 1), with the potency of triC34e being 100- and 40-fold higher than that of C34REG and C34 peptides, respectively. Notably, the triC34e trimer peptide is remarkably more potent in anti-HIV-1 activity than the monomer, indicating that a trimeric form is critical for inhibitory activity. Cytotoxicity from the peptides was not observed at concentrations of 15 μM for C34REG and C34, and 5 μM for triC34e.

We next carried out an assay for the inhibition of viral replication. As shown in Table 2, triC34e showed 30- and 20-fold higher inhibitory activity than peptides C34 and C34REG, respectively. In the two anti-HIV-1 assays, triC34e showed a great enhancement of activity over the C34 monomers. The IC_{50} values obtained in the assays are different, and this can be

Table 1. IC_{50} and CC_{50} values determined by viral fusion inhibition and cell viability assays.

	C34 peptide[a]	C34REG	triC34e
IC_{50} [μM][b]	0.044	0.12	0.0013
CC_{50} [μM][c]	>15	>15	>5

[a] HIV-1 IIIB C34 peptide. [b] IC_{50} values are based on luciferase signals in TZM-bl cells infected with HIV-1 (NL4-3 strain). [c] CC_{50} values are based on the decrease in viability of TZM-bl cells. All data are the mean values from at least three experiments.

Table 2. IC_{50} values determined by inhibition assay based on p24 ELISA.

	C34 peptide	C34REG	triC34e
IC_{50} [μM][a]	1.59	1.06	0.0547

[a] IC_{50} values are based on the production of p24 in MT-4 cells infected with HIV-1 (NL4-3 strain). All data are the mean values from at least three experiments.

explained through differences in experimental procedures. In the fusion inhibition assay, cells were treated with peptides before viral infection. In contrast, in the viral replication inhibition assay, peptides were treated after viral adsorption to cells. Therefore, in the latter case, the infection by HIV-1 might precede peptide binding to gp41.

It has been shown that T-1249, an analogue of enfuvirtide, and its hydrophobic C-terminal region inhibit HIV-1 fusion by interacting with lipid bilayers.[28] The tryptophan-rich domain of T-1249 was shown to play important roles in HIV-1 fusion.[29–31] As enfuvirtide shows weak interaction with the gp41 core structure, and the C34 sequence lacks the C-terminal lipid binding domain, it has been suggested that C34 has a mechanism of action distinct from that of enfuvirtide.[32] Thus, it is of interest to discern the mechanism of the enhanced inhibition observed with triC34e relative to the monomer. Two explanations can be envisaged: 1) the α helicity of the C34 trimer is higher than that of the monomer, as shown in Figure 3 A, and as a result, the C34 trimer binds more strongly to the N36 trimer; and 2) in the mixture with the N36 monomer, the C34 trimer shows less α helicity than its monomer (Figure 3 B). As shown in Figure 3 A, the molar ellipticity at 222 nm is similar for both the C34 trimer and the monomer. Thus, the decrease at 222 nm in the mixture with N36 might be due to a decrease in the α helicity of N36. These results suggest that the C34 trimer might destabilize helix formation in N36 and thus exert potent inhibitory activity. It has been shown that a dimeric C37 (residues 625–661) variant does not show a significant difference in IC_{50} value against HIV-1 from wild-type C37, although the dimeric peptide shows tighter binding to the gp41 N-HR coiled-coil than the C37 monomer.[33] Thus, the mechanism of action of the C34 trimer could be different from that of the dimeric C-peptide. The detailed action mechanism of the trimer as a fusion inhibitor and the reasons behind its remarkable increased anti-HIV-1 activity will be the subjects of future studies in our research group.

A C-terminal helical peptide of HIV-1 gp41 has been designed as a new HIV fusion inhibitor and was synthesized with a novel template and three branched linkers of equal length. The native chemical ligation proceeded by chemoselective coupling in an aqueous medium of an unprotected C34 derivative containing a C-terminal thioester with a three-cysteine-armed scaffold. This process led to the production of triC34e. As a fusion inhibitor, triC34e has potent anti-HIV-1 activity, 100-fold greater than that of the C34REG monomer, although the anti-HIV-1 activity of the N36 trimer is threefold higher than that of the N36 monomer, and the N36 content is the same in both cases.[20] A trimeric form of C34 is evidently critical as the active structure of the fusion inhibitor. The soluble C34 derivative, SC34, retains potent inhibitory effects against enfuvirtide-resistant viruses,[34] and this suggests that the present highly potent trimeric inhibitor could be effective for enfuvirtide-resistant HIV-1 strains. The design of inhibitors that target the dynamic supramolecular mechanism of HIV-1 fusion will be useful for future studies of anti-HIV-1 agents.

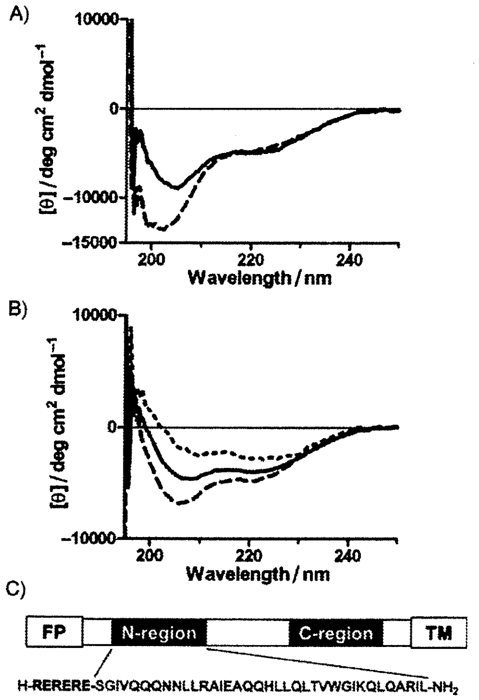


Figure 3. A) CD spectra of C34REG (monomer, -----, 6 μM) and triC34e (trimer, ——, 2 μM). B) CD spectra in the presence or absence of the N36 monomer N36RE:[20] -----, [C34REG (6 μM) + N36RE (6 μM)]; ——, [triC34e (2 μM) + N36RE (6 μM)]; •••••, N36RE (6 μM). In the amino acid sequence of N36RE, the triplet repeat of arginine and glutamic acid is located at the N-terminus of the original N36 sequence.[20] C) Amino acid sequence of N36RE: FP and TM represent the hydrophobic fusion peptide and transmembrane domains, respectively.

Experimental Section

Conjugation of C34REG-thioester and the template to produce triC34e

TCEP·HCl (773 μg, 2.67 μmol) and thiophenol (9 μL, 89 μmol) were dissolved in 0.1 M sodium phosphate buffer (60 μL) containing 6 M urea and EDTA (pH 8.5, 2 mM) under a nitrogen atmosphere. Compound **5** (100 μg, 0.0899 μmol), C34REG-thioester (1.77 mg, 0.297 μmol), and CH_3CN (20 μL) were added. The reaction was stirred for 5 h at 37 °C and monitored by HPLC. The ligation product (triC34e) was separated as an HPLC peak and characterized by ESI-ToF-MS (*m/z* calcd for $C_{703}H_{1108}N_{205}O_{245}S_6$ $[M+H]^+$: 16533.9, found: 16543.8). Purification was performed by reversed-phase HPLC (Cosmosil 5C_{18}-AR II column, 10×250 mm, Nacalai Tesque, Inc.) with elution using a 33–43 % linear gradient of CH_3CN (0.1 % TFA) over 40 min. Purified triC34e, obtained in 17 % yield, was identified by ESI-ToF-MS. Details of the synthesis of these peptides are described in the Supporting Information.

CD spectra

Circular dichroism measurements were performed with a J-720 CD spectropolarimeter equipped with a thermoregulator (Jasco). The wavelength dependence of molar ellipticity [θ] was monitored at 25 °C from λ 195 to 250 nm. The peptides were dissolved in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2).

Virus preparation

For virus preparation, 293FT cells in a 60 mm dish were transfected with the pNL4-3 construct (10 µg) by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 µm filter, and stored at −80 °C as the virus stock.

Anti-HIV-1 assay

For the viral fusion inhibition assay, TZM-bl cells (2×10^{4} cells per 100 µL) were cultured with the NL4-3 virus (5 ng of p24) and serially diluted peptides. After culture for 48 h, cells were lysed, and the luciferase activity was determined with the Steady-Glo luciferase assay system (Promega, Fitchburg, WI, USA).[35] For the viral replication inhibition assay, MT-4 cells (5×10^{4} cells) were exposed to HIV-1 NL4-3 (1 ng of p24) at 4 °C for 30 min. After centrifugation, cells were resuspended with 150 µL medium containing indicated concentrations of serially diluted peptides. Cells were cultured at 37 °C for 3 days, and the concentration of p24 in the culture supernatant was determined by HIV-1 p24 antigen ELISA kit (ZeptoMetrix, Buffalo, NY, USA).

Cytotoxicity assay

The cytotoxic effects of peptides were determined by the CellTiter 96 AQueous One Solution Cell Proliferation assay system (Promega) under the same conditions, but in the absence of viral infection.

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Keywords: antiviral agents · C34 trimers · fusion inhibitors · gp41 · HIV-1

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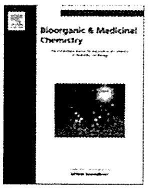

Conjugation of cell-penetrating peptides leads to identification of anti-HIV peptides from matrix proteins

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ABSTRACT

Compounds which inhibit the HIV-1 replication cycle have been found amongst fragment peptides derived from an HIV-1 matrix (MA) protein. Overlapping peptide libraries covering the whole sequence of MA were designed and constructed with the addition of an octa-arginyl group to increase their cell membrane permeability. Imaging experiments with fluorescent-labeled peptides demonstrated these peptides with an octa-arginyl group can penetrate cell membranes. The fusion of an octa-arginyl group was proven to be an efficient way to find active peptides in cells such as HIV-inhibitory peptides.

1. Introduction

Several anti-retroviral drugs beyond reverse transcriptase inhibitors, including effective protease inhibitors[1] and integrase inhibitors[2,3] are currently available to treat human immunodeficiency virus type 1 (HIV-1) infected individuals. We have also developed several anti-HIV agents such as coreceptor CXCR4 antagonists,[4–7] CD4 mimics,[8–10] fusion inhibitors[11] and integrase inhibitors.[12,13] However, the emergence of viral strains with multi-drug resistance (MDR), which accompanies the development of any antiviral drug, has encouraged a search for new types of anti-HIV-1 drugs with different inhibitory mechanisms.

Matrix (MA) proteins are essential for assembly of the virion shell. MA is a component of the Gag precursor protein, Pr55Gag, and is located within the viral membrane.[14,15] It has been reported that MA-derived peptides such as MA(47–59) inhibit infection by HIV,[16] and that MA-derived peptides such as MA(31–45) and MA(41–55) show anti-HIV activity.[17] In addition, Morikawa et al. report that MA(61–75) and MA(71–85) inhibit MA dimerization, a necessary step in the formation of the virion shell.[18] However, the question of whether the above MA peptides can penetrate cell membranes was not addressed in these reports. We speculate that to achieve antiviral activity it is essential that the MA-derived peptides penetrate the cell membrane and function intracellularly. In this paper, we report our design and construction of an overlapping library of fragment peptides derived from the MA protein with a cell membrane permeable signal. Our aim is the discovery of potent lead compounds, which demonstrate HIV inhibitory activity inside the host cells.

2. Materials and methods

2.1. Peptide synthesis

MA-derived fragments and an octa-arginyl (R_8) peptide were synthesized by stepwise elongation techniques of Fmoc-protected amino acids on a Rink amide resin. Coupling reactions were performed using 5.0 equiv of Fmoc-protected amino acid, 5.0 equiv of diisopropylcarbodiimide and 5.0 equiv of 1-hydroxybenzotriazole monohydrate. Ac_2O–pyridine (1/1, v/v) for 20 min was used to acetylate the N-terminus of MA-derived fragments, with the exception of fragment 1. Chloroactylation of the N-terminus of the R_8 peptide, was achieved with 40 equiv of chloroacetic acid, 40 equiv of diisopropylcarbodiimide and 40 equiv of 1-hydroxybenzotriazole monohydrate, treated for 1 h. Cleavage of peptides from resin and side chain deprotection were carried out by stirring for 1.5 h with a mixture of TFA, thioanisole, ethanedithiol, *m*-cresol

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and triisopropylsilane (8.15/0.75/0.75/0.25/0.25/0.1, v/v). After removal of the resins by filtration, the filtrate was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethyl ether. All crude peptides were purified by RP-HPLC and identified by ESI-TOFMS. In the conjugation of the R_8 peptide (or iodoacetamide), the peptide (or iodoacetamide) solution in 0.1 M phosphate buffer, pH 7.8 was added to MA fragments which were synthesized as described above. The reaction mixture was stirred at room temperature under nitrogen. After 24 h (or 1 h for the conjugation of iodoacetamide), purification was performed by RP-HPLC. The purified peptides were identified by ESI-TOF MS and lyophilized. Purities of all final compounds were confirmed to be >95% by analytical HPLC. Detailed data are provided in Supplementary data.

2.2. Anti-HIV-1 assay

Anti-HIV-1 (NL4-3 or NL(AD8)) activity was determined by measurement of the protection against HIV-1-induced cytopathogenicity in MT-4 cells or PM1/CCR5 cells. Various concentrations of test peptide solutions were added to HIV-1 infected MT-4 or PM1/CCR5 cells at multiplicity of infection (MOI) of 0.001 and placed in wells of a 96-well microplate. After 5 day incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The anti-HIV-1 (JR-CSF) activity was also determined by measuring capsid p24 antigen concentrations of the culture supernatant in the infected cultures by a commercially available ELISA assay (ZeptoMetrix Corp., Buffalo, NY).

2.3. CD spectroscopy

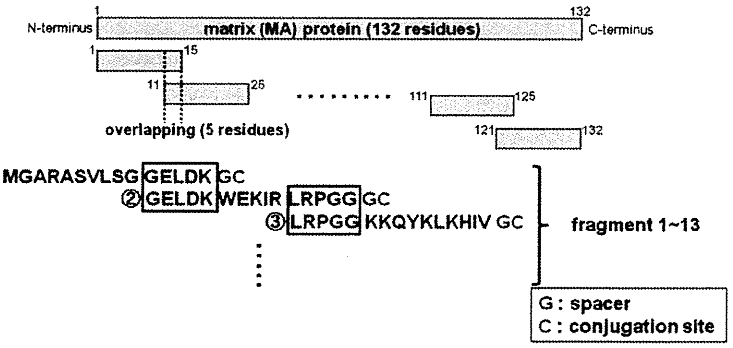
CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25 °C. The measurements were performed using a 0.1 cm path length cuvette at a 0.1 nm spectral resolution. Each spectrum represents the average of 10 scans, and the scan rate was 50 nm/min. The concentrations of samples 8L and 9L were 28.2 and 64.7 μM, respectively, in PBS buffer (pH 7.4).

2.4. Fluorescent imaging of cell-penetrating MA peptides

Cells were seeded on 35 mm glass-bottom dish (2×10^5 cells/dish for HeLa and A549, 1×10^5 cells/dish for CHO-K1) one day before the experiments. The cells were cultured in DMEM/10% FBS/Penicillin–Streptomycin for HeLa and A549, or Ham's F12/10% FBS/Penicillin–Streptomycin for CHO-K1 at 37 °C/5% CO_2. Before the addition of MA peptides, cells were washed with Hanks' balanced salt solutions (HBSS) once. Peptides were added at 5 μM and further cultured for 30 min at 37 °C/5% CO_2. After incubation, cells were washed three times with HBSS and observed under a confocal laser-scanning microscopy (Zeiss LSM510).

3. Results and discussion

An overlapping peptide library spanning the whole sequence of the MA domain, p17, of NL4-3, the Gag precursor Pr55 of HIV-1 was designed. The full sequence of MA consists of 132 amino acid residues. In the peptide library, the MA sequence was divided from the N-terminus in 15-residue segments with an overlap of 5



fragment number	sequence
1	H-MGARASVLSGGELDKGC-NH_2
2	CH_3CO-GELDKWEKIRLRPGGGC-NH_2
3	CH_3CO-LRPGGKKQYKLKHIVGC-NH_2
4	CH_3CO-LKHIVWASRELERFAGC-NH_2
5	CH_3CO-LERFAVNPGLLETSEGC-NH_2
6	CH_3CO-LETSEGSRQILGQLQGC-NH_2
7	CH_3CO-LGQLQPSLQTGSEELGC-NH_2
8	CH_3CO-GSEELRSLYNTIAVLGC-NH_2
9	CH_3CO-TIAVLYSVHQRIDVKGC-NH_2
10	CH_3CO-RIDVKDTKEALDKIEGC-NH_2
11	CH_3CO-LDKIEEEQNKSKKKAGC-NH_2
12	CH_3CO-SKKKAQQAAADTGNNGC-NH_2
13	CH_3CO-DTGNNSQVSQNYGC-NH_2

Figure 1. The construction of MA-based overlapping peptide library.

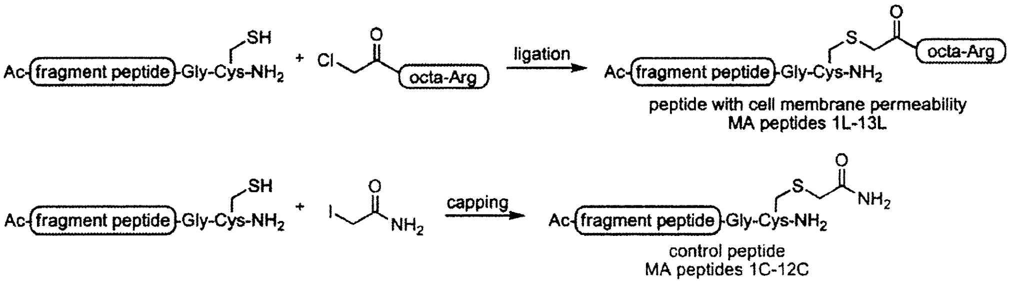


Figure 2. The design of MA peptides with cell membrane permeability (upper) and their control peptides (lower).

residues to preserve secondary structures (Fig. 1). Cys residues of the original MA sequence were changed into Ser residues because of the facility of peptide synthesis. Thirteen MA fragment peptides (1–13) were designed with the addition of Gly as a spacer and Cys as a conjugation site at the C-terminus. To impart cell membrane permeability to these peptides, the N-terminal chloroacetyl group of an octa-arginyl (R_8) peptide[19] was conjugated to the side-chain thiol group of the Cys residue of the above peptides. This resulted in the MA peptides 1L–13L (Fig. 2). R_8 is a cell membrane permeable motif and its fusion with parent peptides is known to produce bioactive peptides with no significant adverse properties.[12,13,20–24] In addition, the R_8-fusion can increase the solubility of MA

Table 1

Anti-HIV activity and cytotoxicity of control MA peptides

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3 (MTT assay) EC_{50}[a] (μM)	NL(AD8) (MTT assay) EC_{50}[a] (μM)	JR-CSF (p24 ELISA) EC_{50}[a] (μM)	(MTT assay) CC_{50}[b] (μM)
1C	>50	ND	ND	>50
2C	17 ± 1.4	1.0	ND	>50
3C	>50	ND	ND	>50
4C	No inhibition at 12.5 μM	ND	ND	14
5C	>50	ND	ND	>50
6C	37 ± 12	24% inhibition at 6.25 μM	25% inhibition at 50 μM	>50
7C	>50	ND	ND	>50
8C	>50	ND	ND	>50
9C	29 ± 1.4	13	8.1	>50
10C	No inhibition at 12.5 μM	ND	ND	17
11C	>50	ND	ND	>50
12C	>50	ND	ND	>50
14C	>50	ND	ND	>50
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and R5-HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the MTT assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain)-induced cytopathogenicity in PM1/CCR5 cells evaluated by the p24 ELISA assay.

[a] EC_{50} values are the concentrations for 50% protection from HIV-1-induced cytopathogenicity in MT-4 cells.

[b] CC_{50} values are the concentrations for 50% reduction of the viability of MT-4 cells. All data are the mean values from at least three independent experiments. ND: not determined.

Table 2

Anti-HIV activity and cytotoxicity of MA peptides with cell membrane permeability

MA peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3(MTT assay) EC_{50} (μM)	NL(AD8)(MTT assay) EC_{50} (μM)	JR-CSF(p24 ELISA) EC_{50} (μM)	(MTT assay) CC_{50} (μM)
1L	30	30	40	>50
2L	21 ± 4.2	>31	ND	32 ± 4.2
3L	no inhibition at 25 μM	ND	ND	36
4L	no inhibition at 3.13 μM	ND	ND	3.7
5L	40	42% inhibition at 50 μM	42	>50
6L	40 ± 8.9	49% inhibition at 50 μM	31	>50
7L	35 ± 1.5	37% inhibition at 50 μM	35% inhibition at 50 μM	>50
8L	2.3 ± 0.3	5.8	7.8	9.0 ± 2.4
9L	2.1 ± 0.5	0.43	0.58	5.7 ± 2.1
10L	43 ± 8.5	42% inhibitionat 50 μM	27	>50
11L	18 ± 3.0	17% inhibition at 25 μM	23	>50
12L	41 ± 5.5	30% inhibition at 25 μM	27	>50
13L	20 ± 2.1	0.43	11	>50
14L	no inhibition at 25 μM	ND	ND	36
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

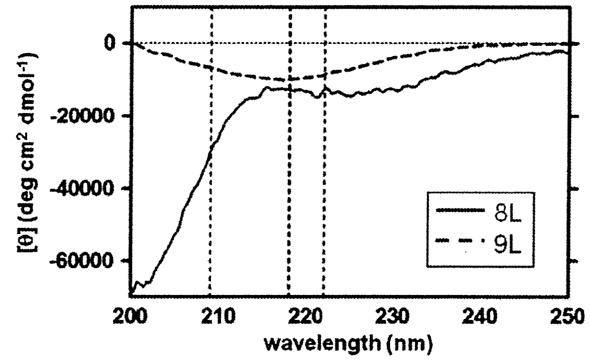


Figure 3. CD spectra of MA peptides 8L (28 μM) and 9L (65 μM) in PBS buffer, pH 7.4 at 25 °C.

peptides whose hydrophobicity is relatively limited. On the other hand, to develop control peptides lacking cell membrane permeability, iodoacetamide was conjugated to the thiol group of the Cys residue to prepare MA peptides 1C–12C (Fig. 2). MA peptide 13C was not synthesized because MA fragment 13 is insoluble in PBS buffer.

The anti-HIV activity of MA peptides 1L–13L and MA peptides 1C–12C, was evaluated. Inhibitory activity against T-cell line-tropic (X4-) HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells and against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM1/CCR5 cells was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, and inhibitory activity against R5-HIV-1 (JR-CSF strain) replication in PM1/CCR5 cells was determined by the p24 ELISA assay. The results are shown in Tables 1 and 2. The control MA peptides 6C and 9C showed slight anti-HIV activity against NL4-3, NL(AD8) and JR-CSF strains, and 2C showed high anti-HIV activity against NL4-3 and NL(AD8) strains, but the other control MA peptides showed no significant anti-HIV activity. 2C showed significant anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains, suggesting that this region of the MA domain is relevant with Gag localization to the plasma membrane (PM)[25] and that 2C might inhibit competitively the interaction between MA and PM. On the other hand, the MA peptides with the exception of 3L and 4L, showed moderate to potent anti-HIV activity against all three strains. These peptides expressed almost the same level of anti-HIV activity against both X4-HIV-1 and R5-HIV-1 strains. The MA peptides 8L and 9L in particular, showed significant anti-HIV activity. These results suggest that MA peptides achieve entry into target cells as a result of the addition of R_8, and inhibit viral replication within the cells. The adjacent peptides 8L and 9L possess an overlapping sequence TIAVL. Such peptides exhibited relatively high cytotoxicity and the MA peptide 4L showed the highest cytotoxicity although it did not show any significant anti-HIV activity. The control MA peptides 1C–12C were relatively weakly cytotoxic. The MA peptides 8C and 9C exhibited no significant cytotoxicity, although the addition of R_8, giving 8L and 9L, caused a remarkable increase in cytotoxicity. This suggests that the octa-arginyl (R_8) sequence is correlated with the

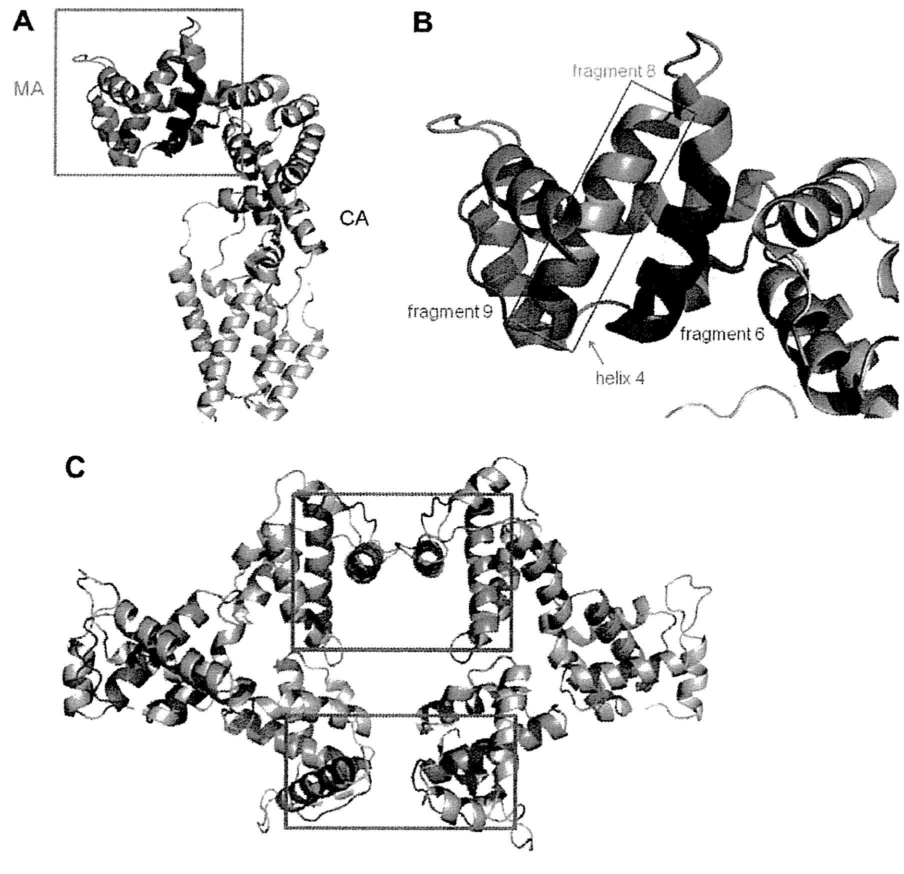


Figure 4. (A) The complete structure of MA and CA proteins (PDB ID: 2gol). (B) The enlarged structure of the highlighted region of (A). (C) The structure of an MA hexamer. Red-colored squares show interfaces between two MA trimers (PDB ID: 1hiw). Orange- and pink-colored helical ribbons represent fragments 8 and 9, respectively.