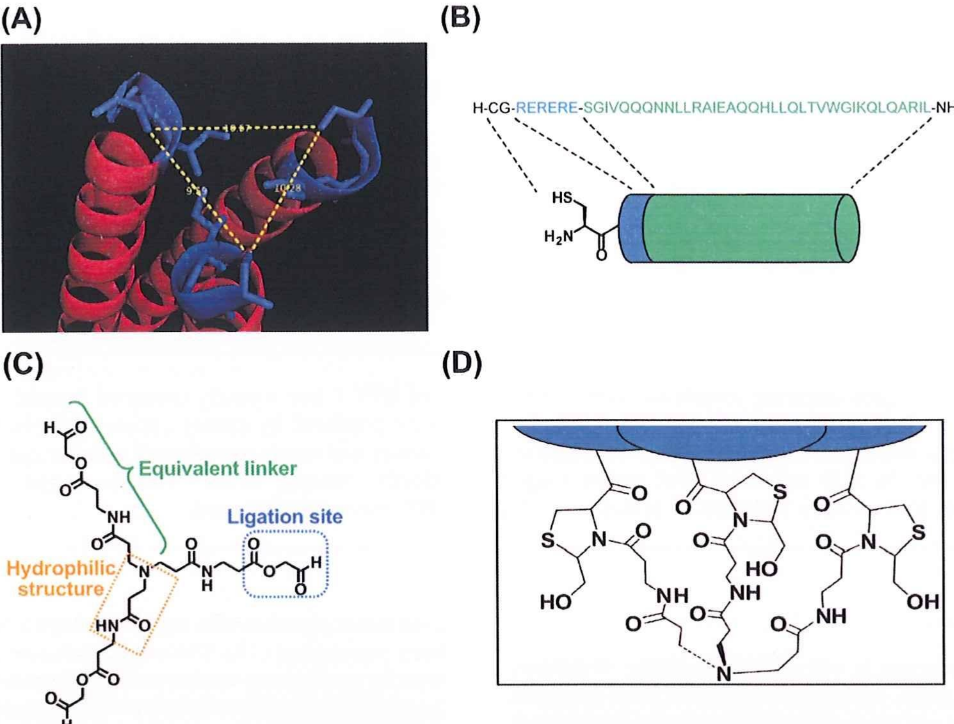

Figure 1. (A) Distances between hydrogen atoms for hydroxyl groups in N-terminal serine residues of N36 helices in trimeric form. The distances were evaluated by PyMOL (*21*). (B) Cartoon presentation of each N36 derived peptide, N36REGC. (C) Design of a C3-symmetric template. The amino acid residues are described in single letters. (D) Conjugated structure of trimeric N36 after thiazolidine ligation.

were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma-Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen was dissolved in 1 μL of DMSO. The solution was mixed with 50 μL of PBS and 50 μL of Freund incomplete adjuvant. The mixture was injected subcutaneously under anesthesia on days 0, 14, 28, 42, and 58. Mice were bled on days 21, 35, 49, and 65. Serum was separated by centrifugation (15 000 rpm) at 4 °C for 15 min and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

Serum Titer ELISA. Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was purchased from Sigma-Aldrich. Antimouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninty-six-well microplates were coated with 25 μL of a synthetic peptide at 10 μg/mL in PBS at 4 °C for overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50 μL of 2-fold serial dilutions of sera from 1/200 to 1/102400 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were washed 10 times with deionized water. Twenty-five microliters of HRP-conjugated antimouse IgG, diluted 1:2000 in 0.02% PBST, was added to each well. After 45 min incubation, the plates were washed 10 times and 25 μL of HRP substrate, prepared by dissolving 10 mg ABTS to 200 μLof HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities were measured at 405 nm.

Virus Preparation. The pNL4-3 construct (8 μg) was transfected into 293T cells by Lipofectamine LTX (Invitrogen, Carlsbad, CA) followed by changing medium at 12 h after transfection. At 48 h after changing medium, the supernatant was collected, passed through a 0.45 μm filter, and stored at −80 °C as HIV-1_{NL4-3} strain before use. For titration, MT-4 cells were infected with serially 3-fold diluted virus from 1/10 to 1/196830, and cultured for 7 days. HIV-1 p24 levels in supernatants were measured, and then the titer of virus solution was calculated.

Anti-HIV Assay. Virus was prepared as described above except that the transfection of pNL4-3 was performed by the calcium phosphate method. Anti-HIV-1 activity was determined on the basis of protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of AZT, N36RE, and triN36e (The starting concentrations are 100, 10, and 1 μM, respectively) were added to HIV-1-infected MT-4 cells (MOI = 0.01) by 2-fold serial dilution and placed in wells of a flat-bottomed microtiter plate (2.0 × 10^4 cells/well). After 5 days' 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 (EC_{50}). Cytotoxicity of compounds was determined on the basis of viability of mock-infected cells using the MTT method (CC_{50}). Each experiment was performed three times independently.

Neutralizing Assay. MT4-cells (1 × 10^5 cells/100 μL) were incubated in 100 μL medium containing 10 μL sera from immunized or preimmunized mice for 1 h at 37 °C, then pretreated MT-4 cells were infected with HIV-1_{NL4-3} (MOI = 0.05). At 3 days after infection, cells were collected by centrifuge at 4000 rpm for 10 min at 4 °C. After discarding supernatant, pellets were lysed with 30 μL of lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% NP-40), then 30 μL of 2 × SDS buffer (125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-ME, 0.004% BPB) were added and boiled for 10 min. The samples (5 μL) were subjected to SDS-page to perform Western blotting. The HIV-1 gag p24 was detected by using Western lightning ECL kit (PerkinElmer, MA) according to manufacturer's instruction after treatment of HIV-1 p24

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Scheme 1. Synthesis of the Equivalently Branched Template 6

EDCI•HCl, HOBt•H_2O, Et_3N, DMF; H_3N–CH$_2$CH$_2$–CO–OBn •*p*-toluenesulfonate **2**; **1** → **3**, 70%

a) Pd/C, H_2 (1 atm), MeOH; b) EDCI•HCl, DMAP, DMF, **4** → **5**, 67%

a) 80% TFA aq.; b) $NaIO_4$, H_2O/MeOH (1:3) → **6**, 42%

antibody (2C2; 1:2000 dilution) (*20*) and anti- mouse IgG (H+L)-HRP (Millipore, MA). The band intensity of p24 was calculated with post/pre-immunized samples by using *ImageJ* image analyzing software.

RESULTS AND DISCUSSION

The N-region of gp41 is known to be an aggregation site involving a trimeric coiled-coil conformation. In design of an N36-derived peptide (N36RE), the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase the solubility in buffer solution (Figure 1B). In order to form a triple helix corresponding precisely to the gp41 prefusion form, we designed the novel C3-symmetric template depicted in Figure 1C. This designed template linker has three branches of equal length and possesses the hydrophilic structure and ligation site for coupling with N36RE. The template was synthesized from the commercially available 3-[bis(2-carboxyethyl)amino]propanoic acid **1** as shown in Scheme 1. Coupling of **1** with β-alanine benzyl ester **2** gave the corresponding triamide **3** in 77% yield. Cleavage of three benzyl esters by hydrogenation and coupling with solketal **4** produced the corresponding triester **5**. Deprotection of the acetonides with aqueous 80% TFA followed by oxidative cleavage of diol group led to the desired template **6**. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N36RE (N36REGC) with a three-armed aldehyde scaffold producing triN36e (Figure 1D). Thiazolidine ligation is a peptide segment coupling strategy which does not require side chain protecting groups (*22*−*26*). The reaction consists of three steps: (i) aldehyde introduction, in which a masked glycolaldehyde ester is linked to the carboxyl terminus of an unprotected peptide by reverse proteolysis; (ii) ring formation, in which the unmasked aldehyde reacts at acidic pH with the α-amino group of an N-terminal cysteine residue of the second unprotected peptide forming a thiazolidine ring; and (iii) rearrangement at higher pH in which O-acyl ester linkage is converted to an N-acyl amide linkage forming a peptide bond with a pseudoproline structure (Figure 2).

Circular dichroism (CD) spectra of triN36e and N36RE, which is a monomer form without N-terminal Cys-Gly residues, are shown in Figure 3A. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, suitable for measurement of CD spectra of membrane proteins (*27, 28*). Both spectra display double minima at 208 and 222 nm and showed high molar ellipticity as absolute values (Table 1). The results indicate that these peptides form a highly structured α-helix and that the helical content of the trimer triN36e is higher than that of the monomer N36RE. Furthermore, to assess the interaction of triN36e with C34, CD spectra of the peptide mixture with C34-derived peptide, C34RE, were measured (Figure 3B,C). The spectrum of triN36e and C34RE mixture showed high molecular ellipticity as an absolute value comparable with that of triN36e alone. This supports the conclusion that C34RE interacts with tri36e and thereby induces a higher helical form as shown previously (*29*).

Mice were immunized with these synthetic gp41 mimetics and antibody production was successfully induced (the detailed titer increase in 5 weeks' immunization is given in the Supporting Information). Two out of three mice showed induction of antibodies against either antigen (N36RE or triN36e). Antibody titers and selectivity of antisera isolated from mice immunized with N36RE or triN36e were evaluated by serum titer ELISA against coated synthetic antigens. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Figure 4). The N36RE-induced antibody showed approximately 5 times higher affinity for N36RE than for triN36e, as 50% bound serum dilutions are 3.88×10^{-4} and 2.14×10^{-3} to N36RE and triN36e, respectively. It is noteworthy that the triN36e-induced antibody showed approximately 30 times higher preference in binding affinity for triN36e antigen than for N36RE (serum dilutions at 50% bound are 3.83×10^{-3} to N36RE and 1.33×10^{-4} to triN36e). Although this evaluation was not determined with purified mAbs, it is clear that the antibodies produced exploit a structural preference for antigens. The mechanism of induction

Template; N36; Imine capture; Cyclization; O,N-Acyl migration

Figure 2. Reaction mechanisms of thiazolidine ligation utilized for assembly of N36RE helices on the template.

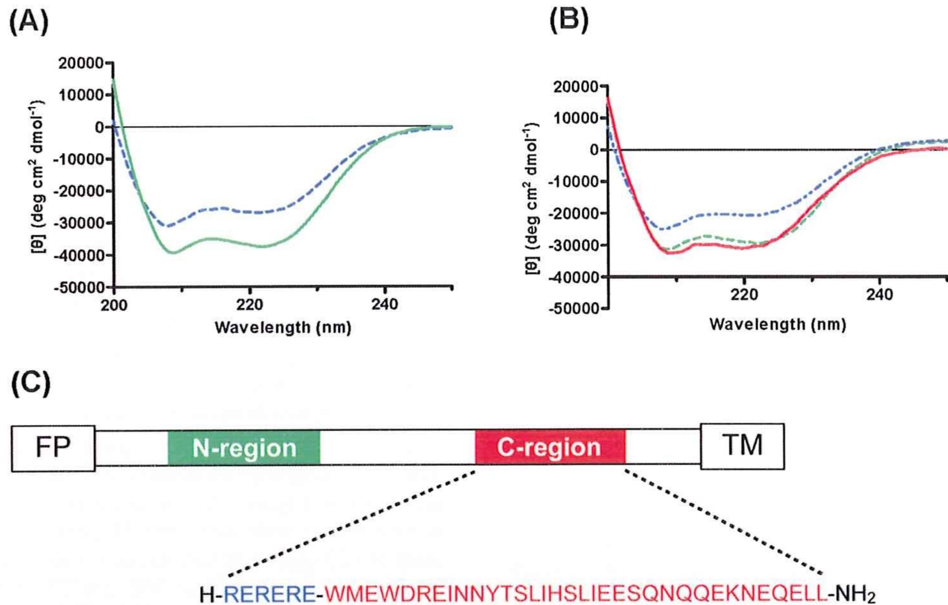

Figure 3. (A) Circular dichroism (CD) spectra of N36RE and triN36e. In the spectra, a blue dashed line and a green line show N36RE (monomer) and triN36e (trimer), respectively. Concentrations of the peptides are 10 and 3.3 μM for N36RE and triN36e, respectively. (B) CD spectra in the presence or absence of C34RE peptide. The spectra show the following: a dashed green line, triN36e; a dashed blue line, C34RE; a red line, triN36e+C34RE, respectively. The concentrations of peptides were as follows: triN36e (2.3 μM), C34-derived peptide C34RE (7 μM), and mixture of both peptides (3.5 μM each). (C) The amino acid sequence of C34RE described in single letters. FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively.

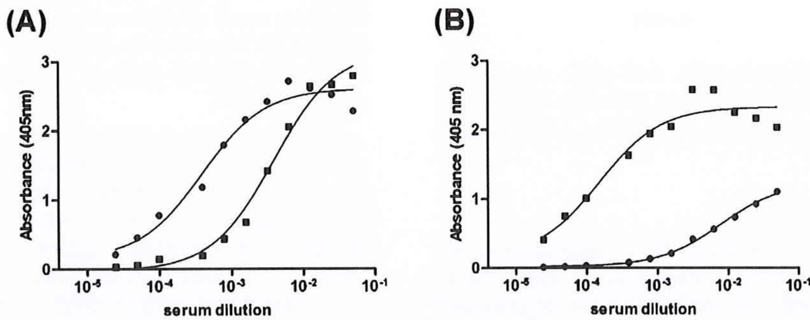

Figure 4. Serum titers of antibodies produced by N36 monomer and conformationally constrained N36 trimeric antigen. The titers were evaluated against N36RE (monomer) (A) and triN36e (trimer) (B). The plots indicate the results of sera obtained from N36RE-immunized mouse (•) and triN36e-immunized mouse (■).

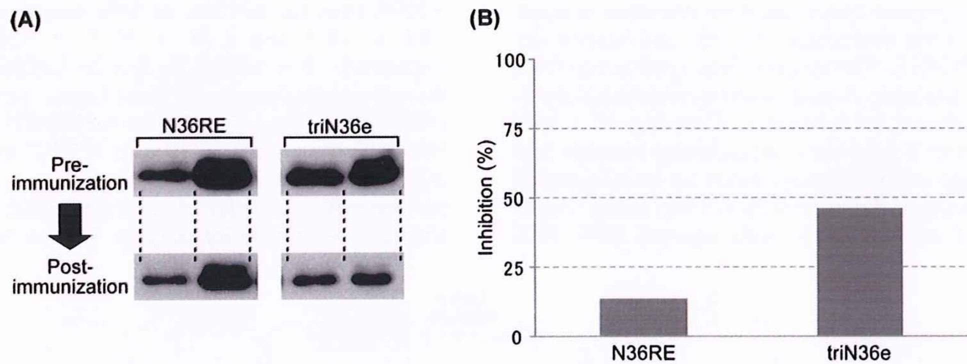

Figure 5. Determination of neutralization activity of the antibodies produced by immunization of peptidomimetic antigens. (A) Results of p24 assay to evaluate inhibition for HIV-1 infection by produced antibodies. Preimmunization sera were used as control. Experiments were duplicated. (B) Average % inhibition of p24 production calculated from the band intensities in panel (A).

of structure-specific antibody is still not clear, but the results could suggest the efficacy of producing antibodies with structural specificity and that the synthesis of structure-involving antigens is an effective strategy when higher specificity is required.

Neutralizing activity of sera against HIV-1 infection was assessed by p24 assays utilizing antisera from two mice that showed antibody production for each antigen (Figure 5). Sera

Table 1. Differences of α-Helicities between N36RE and triN36e Calculated from CD Spectra in Figure 3

	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$	α-helicity
N36RE	−30 957	0.87	73%
triN36e	−38 998	0.96	95%

Table 2. EC_{50} and CC_{50} Values Calculated from Inhibition Assays of Peptidomimetics

	AZT	triN36e	N36RE
EC_{50} (μM)[a]	0.047	0.49	1.4
CC_{50} (μM)[b]	>50	>1	>10

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

from mice immunized with the same antigen showed similar inhibitory activity against viral infection (12.5% and 14.8% for N36RE, 40.3% and 52.1% for triN36e). A trend was observed that the sera from triN36e immunization shows higher inhibition than those from N36RE immunization. This suggests that the synthetic antigen corresponding to the N36 trimeric form induces antibody with neutralization activity superior to that of the monomer peptide antigen and implies a restricted response of B-cells upon immunization to the trimeric form of N36RE. In order to assess the compatibility of induced antibodies in HIV-1 entry inhibition, the HIV-1 inhibitory activities of peptidomimetics (N36RE and triN36e) have been evaluated by viral infection and cytotoxicity assays. A C-terminal region peptide known as Enfuvirtide (T20, Roche/Trimeris) has been used clinically as a fusion inhibitor, and its success indicates that gp41-derived peptides might be potent inhibitors, useful against HIV-1 infection (*30*). In the development of anti-HIV peptides, several mimetics such as Enfuvirtide, CD4 binding site of gp120 (*31*), and protein-nucleic acid interactions (*32*), which disrupt protein−protein interactions, have been produced. As indicated in Table 2, N36 and triN36e showed modest inhibitory activity as reported in previous studies (*33−35*). The potency of triN36e was three times higher than that of N36RE indicating that the active structure of monomer N36RE is a trimeric form. Cytotoxicity of the antigens was not observed at concentrations of 1 μM of triN36e and 10 μM of N36RE.

CONCLUSIONS

In summary, a mimic of HIV-1 gp41-N36 designed as a new vaccine has been synthesized utilizing a novel template with three branched linkers of equal lengths. Thiazolidine-forming ligation attached the esteraldehyde of three-branched template with N-terminal cysteine of peptides in an aqueous medium. The resulting peptide antigen successfully induces antibodies with neutralization activity against HIV-1 infection. It is of special interest that the antibody produced acquires structural preference to antigen, which showed 30 times higher binding affinity for trimer than for monomer. This indicates the effectiveness of the design based on the structural dynamics of HIV-1 fusion mechanism of an antigen which could elicit neutralizing antibodies. In a design based on the N36 region of gp41, the exposed timing of epitopes is limited during HIV-1 entry (*36*), and carbohydrates, which could make accession of antibodies to epitopes difficult, are not associated with the amino acid residues of the native protein. These two advantages could further enhance the potential of a vaccine design based on the N36 region. During preparation of the manuscript, a new HIV vaccine strategy was reported by Burton's group (*37*). The report describes the importance of antibody recognition for the trimer form of surface protein. The trimer-specific antibodies indicate broad and potent neutralization. The gp41 trimer-form specific antibody produced in this study could also obtain the corresponding properties. The elucidation of antibody-producing mechanisms and epitope recognition mode of antibodies in antiserum during HIV-1 entry will be addressed in future studies.

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Supporting Information Available: HPLC chromatograms and NMR charts of compounds **3**, **5**, and **6**. Results of ESI-TOF-MS, and HPLC chromatograms of peptides N36RE, N36REGC, and triN36e. Results of serum titer ELISA of antisera collected during immunization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Synthesis and Evaluation of Artificial Antigen Peptide Based on the Trimeric Form of HIV Fusion Protein

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The trimer formation of the human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein gp41 is necessary for HIV infection. This membrane-fusion mechanism is mediated by the viral Env proteins (gp120/gp41) and receptor on the target cell. Our synthetic peptide antigen has a newly designed template with symmetric linkers mimicking N36 trimeric form. The immunization of the antigen successfully induced antibody against N36. Interestingly, the antibody specifically recognizes the trimeric N36. Our results indicate the strategy of HIV vaccine design based on the native structure of proteins related to HIV fusion mechanisms.

Keywords: antigen, HIV, thiazolidine ligation, vaccine

Introduction

The antibody-based therapy of HIV-1 is a promising treatment of AIDS. In recent years, antibodies for this purpose have been produced by immunization approach and de novo engineering of mAb with molecular evolution tactics such as phage display. Despite enormous efforts, only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. They include gp41 Abs 2F5 and 4E10 and the gp120 Abs 2G12 and b12 [1-3]. The gp41 is a transmembrane envelope glycoprotein. This subunit is devided by the transmembrane region into an endodomain and an ectodomain; the latter contains a hydrophobic amino-terminal fusion peptide, followed by an amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domain with a helical structure (HR1 and HR2, respectively). In membrane fusion process of HIV-1, these subunits form "pre-bundle" complex. The HR1 and HR2 regions are referred as N-terminal helices (N36) and C-terminal helices (C34), respectively. These helices forms six-helix bundle consists of a central parallel trimeric coiled-coil of N36 surrounded by C34 in an antiparallel hairpin fashion in fusion mechanism. In design of immunogens that elicit broadly neutralizing antibodies, one of the strategies is to produce molecules that mimic the mature trimer on the virion surface. These molecules can be recombinant or expressed

on the surface of particles such as pseudovirions or proteoliposomes. The degree of mimicry can be estimated using the broadly neutralizing mAbs; suitable mimics will bind to neutralizing mAbs well but to non-neutralizing mAbs poorly. Based on this approach, the chemically constrained trimeric form of N36 was designed and synthesized. The present trimeric form of N36 has an advantage in term of equivalency of helices. The helices are condensed to the template with equivalent linkers in length. Mice were immunized with the equivalent trimeric form of N36 mimic, and produced antibody indicated the stronger binding affinity for N36 trimer than for N36 monomer. This approach shows the possibility of producing structure-specific Abs by immunization of synthetic antigens corresponding to natural form of viral proteins.

Results and Discussion

<u>*Design and synthesis of trimeric antigen mimics gp41-N36 of HIV-1.*</u>

It has been shown that N36 mimic peptide-antigen involves trimeric coiled-coil of N-region in gp41, which comprises of three N36 derived peptides NP102 and a novel small molecular template for peptide assembly. Several peptide mimics have been synthesized and challenged to neutralization assays [4, 5]. The templates assembling three helical strands contain branched peptide-linkers, which is not completely equivalent in length. The N-region of gp41 is known as a trimeric coiled-coil conformation and as an aggregation site. In design of N36-derived peptide NP102, the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase solubility in buffer. The template was designed to be compatible for hydrophilicity required in thiazolidine ligation [6] with NP103. The three branched linker was designed to be equivalent in length and was elongated by an amide bond for the solubility in buffer utilizing 3-[bis(2-carboxyethyl)amino]propanoic acid as a starting material. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected NP103 with an aldehyde scaffold containing three arms to produce NP104. In this linker design, the equivalency in length would be important for the formation of triplet helix corresponding to the gp41 pre-fusion form (Fig. 1).

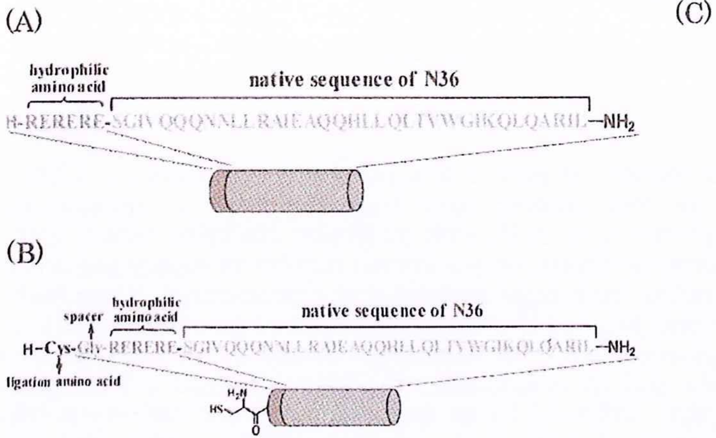

Fig. 1. Amino acid sequences of monomer helix, NP102 (A), and NP103 with unprotected cystein at N-terminal (B). The template is described in (C).

<u>*CD spectra of NP102 and NP104*</u>

CD spectra of NP104 and NP102 displayed double minima at 208 and 222 nm. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, which was previously reported as suitable condition for measurement of CD spectra of membrane proteins. The spectra of NP102 and NP104 peptides showed high molar elipticities ($[\theta]_{220}$ -30,957 and -38,998 deg•cm^2•dmol^{-1}). The results are indicative of a highly α-helical structure of the peptides. Furthermore, to assess the interaction of NP104 with C34, CD spectra of the peptides were measured as follows; NP104 (2.3 μM), C34 derived peptide-C34RE (7 μM) and mixture of both peptides (3.5 μM each). The spectrum of NP104 and C34RE mixture showed comparable molecular eliplicity with NP104 alone. It indicates that C34RE is associated with NP104 and induces higher helical form as shown previously. This result indicated that NP104, a N36 equivalently-constrained trimer, could form six-helix bundle with C34RE corresponding to the native structure of gp41.

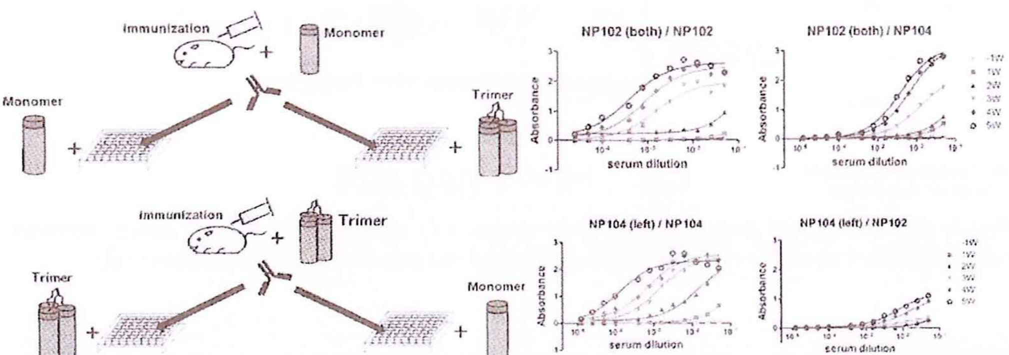

Fig. 2. Immunization and the titer ELISA monitoring induction of antibody. The right panels show specific recognition of NP102 (monomer) and NP104 (tirmer) derived antisera to monomer and trimer antigens.

<u>*Immunization to mice and antibody production.*</u>

Antibody titers and selectivity of antisera from mice immunized with NP102 and NP104 for antigens were evaluated by ELISA against coated synthetic antigens. The results indicated that each antigen has enough antigenicity. To assess the specificity of induced antisera to antigens, the binding to both antigens, NP102 and NP104, was compared (Fig. 2). The NP102-induced antiserum showed approximately 5 times higher affinity for NP102 than for NP104 (50% bound antisera dilutions are 3.84×10^{-4} and 2.14×10^{-3} to NP102 and NP104, respectively). Interestingly, the NP104 induced antiserum showed approximately 30 times higher preference in binding affinity for NP104 antigen than for NP102 (50% bound antisera dilutions are 3.83×10^{-3} and 1.33×10^{-4} to NP102 and NP104, respectively). This evaluation is not dissected with purified mAbs, however, it is clear that produced antibodies exploit structural preference for antigen. The mechanism of induction of structure-specific antibody is still not clear. The results might indicate the efficacy of producing Abs with structural-specificity, and at least the synthesis of structurally-corresponding antigens is one of the effective strategies when higher specificity is required.

<u>*p24 assay*</u>

Neutralizing activity of antisera against HIV-1 was assessed by p24 assay. Three mice are immunized with each antigen (NP102 or NP104). The results of the assays showed two immunized mice produced Abs. Sera induced by the same antigen immunization showed comparable inhibition rates (12.5% and 14.8% for NP102, 40.3% and 52.1% for NP104). The sera from NP104 immunization showed

approximately 4 times higher inhibition rates. This result indicates that the synthetic antigen corresponding to N36 trimeric form is superior to the monomer peptide antigen. The Abs producing mechanism of structural mimetics is unclear, however, this approach caused higher inhibition against HIV-1 infection.

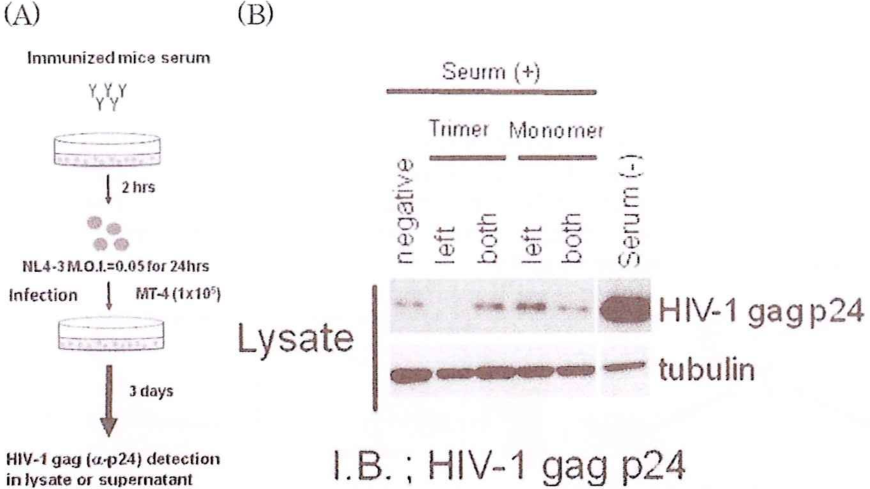

Fig.3. Experimental procedure of p24 assay (A) and results of the assay showing neutralization activity of antiserum derived from trimer-immunized mice (B).

Conclusions

In this study, we synthesized a trimeric peptide antigen mimicking N36 of HIV-1 gp41 using a novel template with three equivalent linkers centering nitrogen as a new vaccine design. The thiazolidine ligation was used to connect esteraldehyde of the three-branched template with N-term cysteine of peptides in aqueous condition. This peptide antigen successfully induced neutralizing against HIV-1 infection. Of special interest in the properties of produced Abs is that the structural-preference to antigen is 30 times higher to the trimeric form than to the monomeric form in binding. Ab-inducing mechanism or recognition mode for fusion mechanism of HIV-1 should be dissected, however, this study indicates the effectiveness of antigen design based on the structural dynamics in HIV-1 fusion mechanism.

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From Reverse to Forward Chemical Genomics: Development of Anti-HIV Agents

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To date, several anti-HIV agents have been developed based on reverse chemical genomics, in which target molecules are fixed. We found that CD4 mimic compounds have a great effect on the dynamic supramolecular mechanism of HIV entry, and that their combinational use with CXCR4 antagonists or with neutralizing antibodies shows a synergistic effect. On the other hand, based on forward chemical genomics, in which active compounds are searched according to the screening of random libraries, effective leads such as integrase inhibitors and matrix peptides have been found.

Keywords: CD4 mimic, forward chemical genomics, integrase inhibitor, matrix peptide, reverse chemical genomics

Introduction

Recently, highly active anti-retroviral therapy (HAART), which involves a combinational use of reverse transcriptase inhibitors and HIV protease inhibitors, has brought us a great success in the clinical treatment of AIDS patients. However, HAART has serious clinical problems including the emergence of multi-drug resistant HIV-1 strains. These drawbacks encouraged us to find novel drugs and increase repertoires of anti-HIV agents with various action mechanisms. The recent disclosing of the dynamic supramolecular mechanism in HIV-entry has provided potentials to find a new type of drugs. To date, we have synthesized HIV-entry inhibitors, especially coreceptor CXCR4 antagonists. In the present study, CD4 mimics in consideration of synergic effects with other entry inhibitors or neutralizing antibodies were developed. The development of the above anti-HIV agents is based on the concept of reverse chemical genomics, in which target molecules are fixed. On the other hand, based on the concept of forward chemical genomics, in which active compounds are discovered according to the screening of random libraries, effective peptide leads such as integrase inhibitors and matrix (MA) peptides were searched.

Results and Discussion

According to the concept of reverse chemical genomics, CD4 mimic compounds were developed based on NBD-556 [1, 2] (Fig. 1). Several compounds caused conformational change of an HIV surface protein, gp120. FACS analysis showed that in the presence of several compounds the anti-V3 antibody strongly binds to envelope-expressing cells. In addition, a synergistic effect of CD4 mimic compounds with an anti-V3 antibody

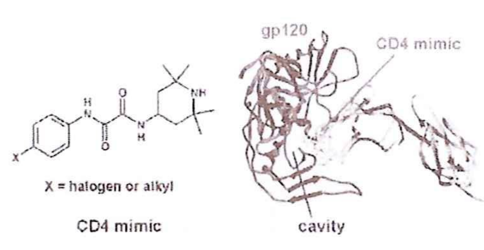

Fig. 1. Structures of CD4 mimic compounds (left) and the complex of gp120 and CD4 (right).

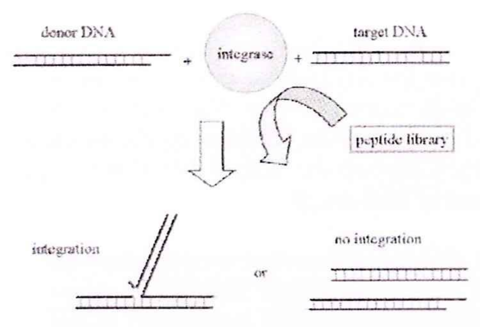

Fig. 2. Integrase inhibition assay using random peptide libraries.

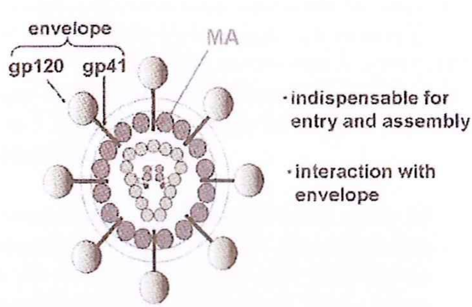

Fig. 3. Matrix (MA) in HIV particle.

or with a CXCR4 antagonist T140 was observed. According to the concept of forward chemical genomics, anti-HIV lead compounds were searched by the screening of random peptide libraries based on HIV-1 proteins. First, potent peptide leads were found by an integrase inhibition assay [3]. Second, effective leads were found in overlapping peptide libraries of MA.

As such, from a point of view on chemical biology, anti-HIV leads have been found utilizing reverse and forward chemical genomics. Furthermore, antibody-based therapy is still thought to be a promising treatment for AIDS. These anti-HIV agents might be important and useful compounds in consideration of cocktail therapy of AIDS. In addition, the present concept of chemical biology for the development of anti-HIV agents would be essential for drug discovery in medicinal chemistry.

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Caged DAG-Lactones for Study of Cellular Signaling in a Spatial- and Temporal-Specific Manner

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Protein kinase C (PKC) plays pivotal roles in cellular signal transductions. In order to elucidate PKC signaling mechanisms in detail, it is useful to develop functional molecules which can activate PKC in a spatial- and temporal-specific manner. We developed caged diacylglycerol (DAG)-lactones which can activate PKC by photoirradiation. Based on the photo-activation mechanism, the binding of DAG-lactones to PKC in cells was controlled.

Keywords: caged compound, diacylglycerol (DAG)-lactone, protein kinase C

Introduction

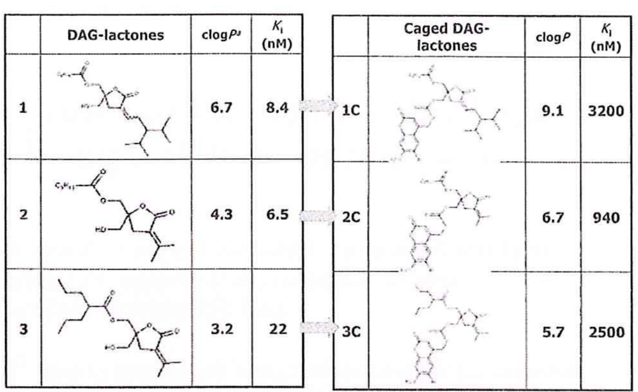
The signal transduction pathways attract a great interest especially in the interaction with small organic compounds in the field of chemical biology. To assess the phenomena induced by bioactive compounds, the addition of compounds could not be a sufficient way to observe the biological activity because the effects would be the whole cell scale and the signals would be multiple combined effects. To solve these problems, chemists have been developed "caged" compounds, which is not active when the pharmacophore is blocked by the photo-activatable molecules. Triggered by photo irradiation, which would be very limited ROI in the cell, the spatial- and temporal-specific effects of compounds would be observed. Several "caging" compounds have been developed and these molecules have their own advantages. In this study, we utilized the coumarin-based "caging" molecules, namely Bmc and Bhc" [1], to block the binding of DAG-lactones [2] to protein kinase Cδ [3] to assess their effects on translocation in cytoplasm.

Results and Discussion

Caged DAG-lactones 1-3 were successfully synthesized. The hydroxyl group of DAG-lactones which is a critical pharmacophore moiety was protected by a 6-bromo-7-methoxycoumarin-4-ylmethoxycarbonyl (Bmcmoc) group. The Bmcmoc group is a suitable phototrigger for alchols with high photochemical efficiency. To

evaluate the effect of caged groups on PKC binding of compounds, binding assay was performed by competition assay with [^{3}H]-PDBu. The results indicated the hydroxyl group is important for PKC binding as described previously. The loss of binding affinity was ranged from 110- to 400-folds (Fig. 1).

Photolysis reaction of caged DAG-lactones by UV irradiation (350 nm) was monitored by HPLC analysis to find generation of DAG-lactones. The results revealed that the time to reach 90% conversion ($t_{90\%}$) of compounds **1** and **2** are about 6 min and 5 min, respectively. To further assess the properties of caged DAG-lactones, confocal laser microscopy analyses were utilized. Activation of PKC by exogenous ligands can be analyzed by its translocation in cells. The GFP-tagged PKC was expressed transiently in a CHO cell line. The addition of caged DAG-lactones showed no change in location of PKC in cells. The results correspond to the kinase activity of these compounds. On the contrary, the addition of DAG-lactones without a caging group showed clear translocation of PKC. These results indicate that photolysis of caged DAG-lactones could be a trigger of activation of PKC translocation. To assess the control of activation in spatial- and temporal manner, the area of photoirradiation was limited in a part of target cells. In this experiment, Bhc-protected DAG lactone 2, which showed faster activation of PKC, was utilized. The results showed clear translocation of PKC in the cell (Fig. 2).



	DAG-lactones	clogP[a]	K_i (nM)		Caged DAG-lactones	clogP	K_i (nM)
1		6.7	8.4	1C		9.1	3200
2		4.3	6.5	2C		6.7	940
3		3.2	22	3C		5.7	2500

Fig. 1. Binding assays of caged DAG-lactones 1-3. The structures of compounds are shown in columns. [a]computed logP.

It has been shown that caging technology is a powerful tool to investigate cellular functions. Our results indicate this technology could be applied to exogenous ligands of protein kinase C. The present results indicate that the activity of DAG-lactones can be controlled in a spatial- and temporal-manner by photo-removable protecting groups. This method would be a useful tool for elucidation of activation mechanism of PKC in mammalian cells.

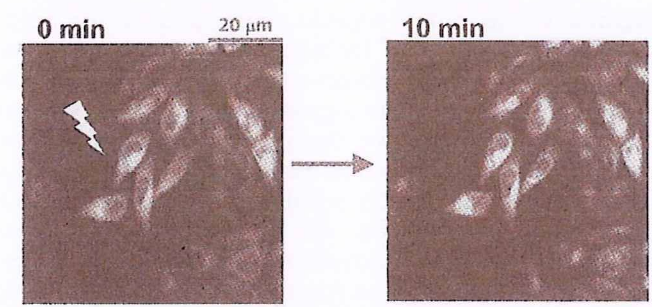


Fig. 2. The photoirradiation on the limited cell region. The circle in the center shows region of interest (ROI).

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Fluorescent-Based Orthogonal Sensing Methods for Double Evaluation in PKC Ligands Screening

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Protein kinase C (PKC) is proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. To find drug lead compounds, development of screening methods, which would be applicable to high-throughput screening, is critical. In this study, we developed two screening methods based on fluorescent labeling to PKC ligand binding domain (C1 domain) and DAG-lactone.

Keywords: DAG-lactone, liagnd-screening, fluorescent labeling, PKC C1 domain

Introduction

Protein kinase C (PKC) is a family of enzymes for phosphorylation, which is specific for Ser and Thr residues. PKC family comprises at least 11 isozymes, which play fundamental roles in signaling pathways that regulate cell cycle progression, differentiation and apoptosis [1]. PKC has also been proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. In PKC activation that depends on such as a diacylglycerol (DAG) and phorbol ester (tumor promoter), C1 domain plays a critical role in these ligands binding. To evaluate ligand binding to C1 domain, two different approaches utilizing fluorescent sensing were developed instead of conventional method using radioisotopes. The first method utilizes the synthetic δC1b domain derivatives bearing a dansyl group, which is environmentally responsive (Fig. 1, left). The second method involves competitive ligand

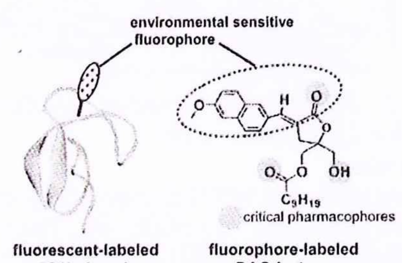

Fig. 1. Orthogonal fluorescent screening tools.

replacement by a fluorophore-labeled DAG-lactone (Fig. 1, right). DAG-lactone is a synthetic PKC ligand based on DAG. To reduce the entropic penalty of DAG, the glycerol backbone is constrained by forming a lactone ring [2]. In this study, the fluorescent screening tools, differently labeled on the acceptor and the ligand, were applied for evaluations of binding affinity of PKC ligands.

Results and Discussion

For the C1b domain of PKCδ (δC1b) (221-281), amino acids at three positions near the binding pocket were selected as insertion sites of fluorescent dye (Ser 240, Thr 242) [3]. A dansyl group was adopted as a fluorescent dye based on its sensitivity to environmental change and small molecular size. To introduce the dansyl group by Fmoc-SPPS, Fmoc-Lys(dansyl-Gly)-OH was prepared. Each fluorescent-labled δC1b domain analog was successfully synthesized. Ligand bindings of these fluorescent analogs were evaluated by utilizing [^{3}H]-phorbol 12,13-dibutylate (PDBu). The two δC1b analogs, T242K(dansyl-G) and S240K(dansyl-G), showed similar binding affinity with that of wild-type. In titration experiments of ligands, the increase of fluorescent intensity and the shift of fluorescence emission maxima showed reasonable correspondence to the binding affinity of ligands for PKC evaluated by the RI method (Fig. 2a).

As a new probe of PKC ligands binding assay based on the competitive inhibition, a fluorophore-labeled DAG-lactone was synthesized. The lactone has environmentally sensitive fluorophore on the position which is not necessary for binding to PKC C1 domain. The binding affinity of a synthetic fluorophore-labeled DAG-lactone for the PKCδ was evaluated by utilizing [^{3}H]-PDBu. The environmental sensitivity of the synthetic DAG-lactone derivative was confirmed by fluorescent measurement in various solvents. The fluorescent intensity of the fluorophore-labeled DAG-lactone was increased by binding to PKC, which indicates the hydrophobic environment of the binding pocket. According to an increase in the concentration of a test compound, the fluorescence intensity was decreased, indicating replacement of the fluorophore-labeled DAG-lactone. The fluorescent spectra in titration of PDBu are shown in Fig. 2b. The IC_{50} values were obtained from the curve-fitting of titrations of known compounds. The fluorescent-based inhibition assay showed a positive correlation between IC_{50} values and K_i values in the RI assay.

In summary, novel screening tools for PKC ligands based on fluorescent-labeling of C1b domain and DAG-lactone have been successfully developed. The combinational use of these fluorescent-labeling methods would lead to detailed and reliable evaluation of ligand compounds for PKC ligands, wchich does not require washing steps.

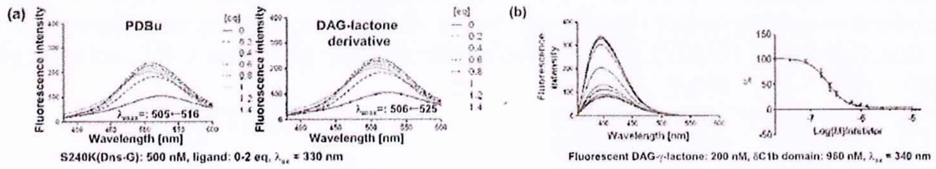

Fig. 2. Fluorescent titration of fluorescent-labeled δC1b (a) and fluorophore -labeled DAG-lactone (b).

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O-26

光機能性リガンドを用いたプロテインキナーゼＣの活性化制御

Spatio-temporal Activation of Protein Kinase C by a Photoactivatable Ligand

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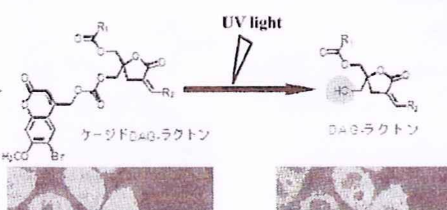
プロテインキナーゼ C（PKC）はジアシルグリセロール（DAG）をセカンドメッセンジャーとするセリン・スレオニン特異的リン酸化酵素であり，がんやアルツハイマー病の治療薬創製の標的酵素として注目されている。演者らはケージド基で保護した PKC 特異的リガンドを創製し，紫外光照射による PKC 活性化の時間・空間的な制御を試みた。DAG を環化することによって結合活性を上昇させた DAG–ラクトンの重要なファーマコフォアである OH 基を 6-Bromo-7-methoxycoumarin（Bmc）（光分解性保護基）により保護したケージド DAG–ラクトン誘導体を合成した。ケージド DAG–ラクトンを緩衝液中で紫外光照射し，Bmc 基の脱保護，及び DAG–ラクトンの出現を HPLC 分析により確認した。また，その結果から分解反応の量子収率等を算出した。ケージド DAG-ラクトンの PKCδ活性化能について，試験管内での ^{3}H[PDBu]（ホルボールエステル）との競合阻害活性，リン酸化アッセイ，および CHO-K1 細胞内における GFP 融合 PKCδの細胞内局在変化（図）によって検討した。その結果，ケージド DAG ラクトンはいずれの場合も PKCδに対する結合活性，活性化能を持たず，紫外光照射によってケージド基を脱保護した場合においてのみ PKCδに対する結合活性を出現させ，活性化も行うことが確認された。以上のことから，ケージド DAG–ラクトンへの紫外光照射による脱保護，それに伴う結合活性の回復を用いて PKCδの活性化を時間・空間的に制御できる可能性が示された。



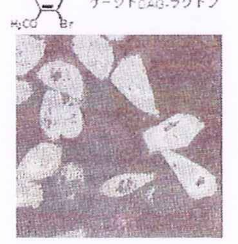


図. DAG-ラクトンのケージド化（PKC に対する結合活性に重要な水酸基を保護）と光照射による再活性化

P-35*

蛍光性 diacylglycerol-lactone 誘導体の合成と機能評価

Synthesis and Evaluation of Fluorescent Diacylglycerol-lactone Derivatives

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Protein kinase C (PKC)は、セリン/スレオニン特異的なリン酸化酵素であり、細胞内シグナル伝達系において重要な役割を果たしている。PKC が持っている C1b ドメインは、シグナル伝達系のセカンドメッセンジャーとしてはたらく diacylglycerol (DAG)の結合標的部位である。この C1b ドメインは、腫瘍プロモーターの結合部位でもあり、がん治療薬創製のターゲットとして注目されている。

当研究室では、Marquez らによって開発された方法に基づき、天然のリガンドである DAG の誘導体を環化することにより、DAG-γ-lactone 誘導体を合成している。これらの PKC への結合活性は、放射性同位体(RI)でラベル化された[20-^{3}H]PDBu をプローブとした競合結合阻害アッセイを用いて評価している。

本研究では、DAG-lactone 誘導体の R_1 または R_2 に蛍光基を導入した蛍光性 DAG-lactone 誘導体を合成し、[20-^{3}H]PDBu に代わる競合プローブの開発を行った(Fig.1)。この蛍光性 DAG-lactone が PKC-C1b ドメインとの結合に伴って蛍光変化を生じた(Fig.1a)ので、その結果リガンド候補品の競合的結合を蛍光変化として検出することができた (Fig.1b)。この方法では、RI アッセイでは必要な余剰プローブ除去操作が不要あり、[20-^{3}H]PDBu にかわる有用なスクリーニングプローブとして期待される。

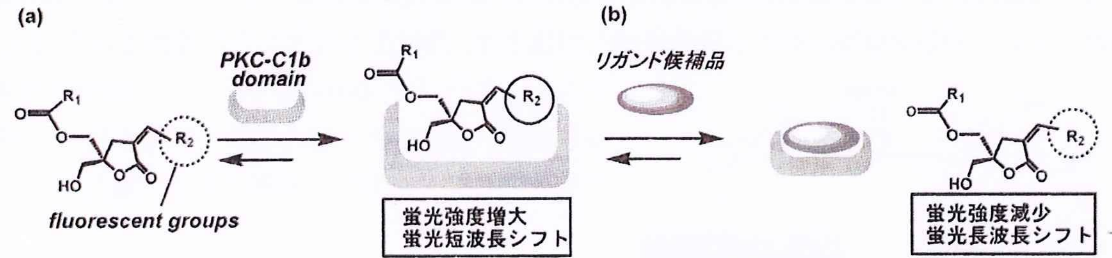

Fig. 1. (a)蛍光性DAG-lactoneとPKC-C1bドメインとの結合
(b)蛍光性DAG-lactoneに対するリガンド候補品の競合的結合

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P-39

フォワードケミカルジェネティクスを応用した HIVインテグラーゼ阻害剤の創製

Development of HIV Integrase Inhibitors Based on Forward Chemical Genetics

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AIDS 治療薬としては逆転写酵素阻害剤やプロテアーゼ阻害剤を 2, 3 剤併用する療法 HAART が成果をあげているが、耐性ウイルスの出現や副作用などの問題により新規の阻害機序を持つ薬の開発が急務となっている。そういった中で、膜融合阻害剤やコレセプターCCR5 の阻害剤、インテグラーゼ阻害剤等も登場してきており、抗エイズ薬のレパートリーは年々着実に増えてきおり、これらはエイズ患者の延命効果に貢献すると思われる。これまで開発されてきた抗エイズ薬はターゲット設定型の創薬研究でうまれたものがほとんどである。そこで我々は、今までの概念を全く切り換えて、フォワードケミカルジェネティクスを活用し、ランダムライブラリーから目的活性を持った化合物、すなわち抗エイズ薬を見つけるという手法を応用した。その結果、今回ペプチド性インテグラーゼ阻害剤を HIV の遺伝子産物から見出すことに成功した。

我々は、HIV 遺伝子産物であるタンパク質由来のアミノ酸配列をもとにしたオーバーラッピングペプチドライブラリー（アミノ酸 10～17 残基）から、in vitro において阻害活性を有する化合物を探索した。その結果、HIV 自身が有するアクセサリータンパク質である Vpr 由来の部分ペプチドライブラリーからインテグラーゼ阻害ペプチドを同定した。そこで、インテグラーゼは細胞内で作用するので、これらのペプチドに細胞膜透過モチーフペプチドである octa-arginine を付加することで細胞膜透過性を付与させ、cell を用いた抗 HIV 活性の検討も行った結果、HIV 複製を抑制した。今回はさらにアミノ酸置換を含む構造活性相関によりさらに高活性ペプチドを得ることができた。このペプチドは新たな抗 HIV 治療薬のリード化合物として期待できる。

P-89*

新規タグ－プローブシステムの開発と タンパク質蛍光イメージングへの応用

Development and Application of a New Tag-Probe System for the Fluorescent Imaging of Proteins

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緑色蛍光タンパク質（Green Fluorescent Protein: GFP）に代表される蛍光プローブは、細胞中のタンパク質を蛍光イメージングするためのツールとして非常に有用である。近年、遺伝子工学的に標的タンパク質にタグとなるペプチドあるいはタンパク質を付加し、タグ特異的に結合する蛍光性プローブ分子を用いて標的タンパク質を蛍光ラベル化する方法が新たに提唱され、さまざまなタグ－プローブ分子ペアの開発が精力的に行われている。これらのタグ－プローブペアは、翻訳後の任意の時間に種々の蛍光プローブ分子を作用させることにより標的タンパク質の時空間的な「染め分け」を可能とすることから、パルスチェイス実験などにおいて有用なツールとなると期待されている。我々は、これまでにロイシンジッパーペプチドの特性を利用し、タグとの結合に伴ってプローブの蛍光波長および蛍光強度が顕著に変化する新規の蛍光変化型タグ－プローブペア（ZIP タグ－プローブペア）の開発を行ってきた。ZIP タグ－プローブペアはループにより連結した逆平行 2 本鎖のα-ヘリックスペプチドタグと、親水/疎水の環境に応答して蛍光波長と蛍光強度が変化する蛍光色素である 4-Nitrobenzo-2-oxa-1,3-diazole (NBD)を有する 1 本鎖α-ヘリックスペプチドプローブから構成されている。蛍光滴定実験の結果、この ZIP タグ－プローブペアは抗原－抗体反応に匹敵する結合親和性を有し、3 本鎖のロイシンジッパー構造を形成したときに NBD がロイシンジッパー構造内部の疎水性コアに選択的に配置されることにより、30 nm 以上の蛍光波長シフトと 18 以上の蛍光強度の増大を示す。これによりタグ－プローブ複合体と遊離のプローブの識別が容易になり、ZIP タグ－プローブペアは洗浄操作が不要なタンパク質イメージングツールであると考えられる。本研究では ZIP タグ－プローブペアを用い、膜タンパク質の一つである CXCR4 の蛍光イメージングに成功したので報告する（図 1）。また、NBD に代わる蛍光色素として 7-Diethylaminocoumarin-3-carboxylic acid (DEAC)を導入した ZIP タグ－プローブペアは、結合に伴って 50 倍以上の蛍光強度増大を示すことも明らかとなったので、これらの詳細について本発表であわせて報告する。

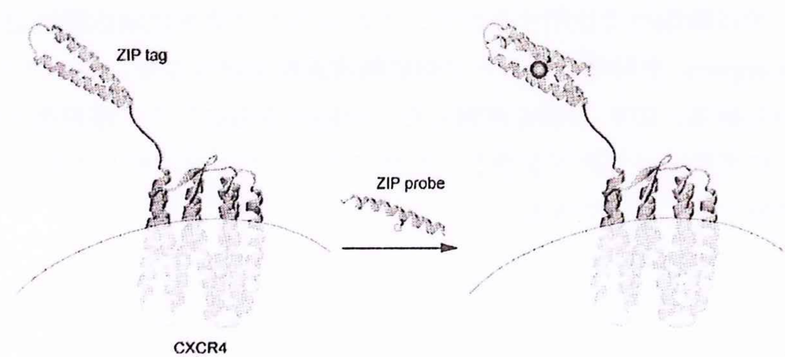

図 1　ZIP タグ－プローブペアを用いた CXCR4 の蛍光イメージング

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P-6 遺伝子機能制御に向けたプログラム可能な DNA メチル化酵素の創製
Development of a programmable DNA methylase toward targeted gene silencing

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○ Wataru Nomura[1], Akemi Masuda[1,2], Tsuyoshi Okuda[1,2], Carlos F. Barbas, III[3], Hirokazu Tamamura[1,2] ([1]Institute of Biomaterials and Bioengineering, Tokyo Med. Dent. Univ., [2]School of Biomed. Sci., Tokyo Med. Dent. Univ., [3]Dept. Mol. Biol., The Scripps Res. Inst.)

シトシン塩基のメチル化はヒストンの脱アセチル化を促進し、それによってクロマチン構造変化が誘起され、遺伝子発現の抑制を行う。DNA メチル化パターンは細胞の世代間をまたいで再生されるため、DNA のメチル化によって永久的な遺伝子発現の抑制が可能となる。そのため、特定の標的 DNA でのメチル化制御は、その標的遺伝子を抑制するために非常に有効な手段となり得る。これまで、DNA メチル化酵素と亜鉛フィンガーモチーフを融合することで、配列特異的な DNA メチル化を行う試みがなされてきた。しかし、DNA メチル化酵素に由来する DNA 認識能によって、非特異的なメチル化が高い頻度で観察されることが問題であった。

本研究では、酵素ドメインを二分割し、それぞれを亜鉛フィンガードメインとの融合体とする分割型 DNA メチル化酵素をデザインした。この酵素では、各亜鉛フィンガードメインがメチル化標的配列の両端にそれぞれ結合する。それによる近接効果によって酵素ドメインが再会合して標的配列がメチル化されると考えた。構築した分割型酵素を大腸菌内で発現させ、その DNA メチル化機能を HhaI 制限酵素切断、Bisulfite シークエンス法などによって解析した。その結果、標的遺伝子上の CpG 配列のみが高い特異性をもってメチル化されることが明らかになった。

本研究結果は、標的遺伝子配列に特異的な DNA メチル化反応、及び亜鉛フィンガーモチーフによる標的 DNA での酵素ドメインの再会合が in vivo で行われた初の例であり、今後の哺乳類細胞内でのメチル化反応への応用が期待される。