

## ORIGINAL ARTICLE

# The high mannose-type glycan binding lectin actinohivin: dimerization greatly improves anti-HIV activity

Atsushi Takahashi<sup>1</sup>, Junji Inokoshi<sup>2</sup>, Atsuko Hachiya<sup>3</sup>, Shinichi Oka<sup>3</sup>, Satoshi Omura<sup>4</sup> and Haruo Tanaka<sup>1,5</sup>

The actinomycete-derived lectin actinohivin (AH) inhibits entry of HIV-1 to susceptible cells at low nM concentrations. The cooperative binding of three segments of AH to three high mannose-type glycans (HMTGs) of HIV-1 gp120 generates specific and strong anti-HIV activity. Dimerization of AH effectively improves anti-HIV activity by increasing the number of HMTG-binding pockets. AH dimers were prepared using an *Escherichia coli* expression system and their anti-syncytium formation and anti-HIV activities were evaluated. Each dimer was constructed by a head-to-tail fusion of two AH molecules, with or without a spacer. As a result, His-TEV-AH/RTB(132–143)/AH, which has the residues 132–143 of ricin toxin B-chain (RTB) as a spacer, had 20-fold higher anti-syncytium formation activity and also exhibited 2–30-fold higher anti-HIV activity than AH against various clinically isolated HIV-1 strains, including drug-resistant ones. Mutation analysis implies that all six HMTG-binding pockets of the dimer participated in HMTG binding. Several AH dimers with different spacer sequences showed diverse activities, suggesting that the spacer sequence is an important factor to create higher anti-HIV activity. A dimer with improved anti-HIV activity would be a good candidate for investigation as a potential microbicide to prevent HIV transmission.

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**Keywords:** actinohivin; anti-HIV; dimer; high mannose-type glycan; mannose-binding lectin

## INTRODUCTION

The first step in HIV-1 infection is entry into cells initiated via binding of the HIV-1 envelope glycoprotein gp120 to cellular receptors, namely CD4 and co-receptors (CXCR4 or CCR5).<sup>1,2</sup> Through induced-conformational changes of gp120, gp41 which is non-covalently bound to gp120, is exposed so that the N-terminus end of gp41 is inserted into the cellular membrane as a trigger of virus–cell membrane fusion enabling infection.<sup>3–5</sup> Therefore, HIV entry inhibitors can provide an alternative approach toward anti-HIV therapy, although development of anti-HIV drugs has predominantly focused on inhibitors of HIV reverse transcriptase, protease and integrase.

HIV gp120 is highly glycosylated. Approximately 50% of its MW is because of N-linked glycans consisting of complex-, hybrid- and high mannose-type glycans (HMTGs).<sup>6</sup> These glycans contribute to low antigenicity of gp120 by hiding epitopes crucial for HIV infectivity.<sup>7</sup> Recently, the glycan-targeting lectins produced by various species have received increasing attention for their HIV infection inhibitory effects. Carbohydrate-binding properties and anti-HIV activities of several lectins, such as algae-derived cyanovirin-N and griffithsin, and plant-derived *Galanthus nivalis* agglutinin, have been well studied<sup>8–10</sup> to reveal their bifunctional inhibitions of not only cell-free virus

infection, but also syncytium formation between virus-infected and uninfected cells. Furthermore, there is a profound interest in what their carbohydrate recognition is commonly distinctive to mannose oligomers such as Man- $\alpha$ (1–2)-Man or Man- $\alpha$ (1–3)-Man of HMTGs. Therefore, lectins targeting the glycans, especially HMTGs of gp120, are becoming attractive candidates for development of an anti-HIV drug, as well as a topical microbicide<sup>11</sup> preventing HIV transmission.

Actinohivin (AH), an actinomycete-derived anti-HIV lectin, inhibits not only infection of laboratory and clinically isolated-HIV strains, but also T- and M-tropic syncytium formation.<sup>12–15</sup> The broad range of anti-HIV activity of AH is brought about by its specific interaction with Man- $\alpha$ (1–2)-Man units of HMTGs.<sup>13,16</sup> AH consists of 114 amino acid residues and its primary sequence is divided into three segments (the residues 1–38, 39–76 and 77–114 for the segments 1, 2 and 3, respectively) containing well-conserved LD and QXW motifs, respectively. The tertiary structure of AH<sup>16</sup> shows that three segments assemble in a similar manner to  $\beta$ -trefoil fold.<sup>17</sup> Based on these profiles, AH is classified into the carbohydrate-binding module family 13.

Proteins belonging to carbohydrate-binding module family 13 generally possess one or several units of  $\beta$ -trefoil fold, of which each

<sup>1</sup>Faculty of Pharmacy and College of Science and Engineering, Iwaki Meisei University, Iwaki, Fukushima, Japan; <sup>2</sup>School of Pharmacy, Kitasato University, Minato-ku, Tokyo, Japan; <sup>3</sup>AIDS Clinical Center, International Medical Center of Japan, Shinjuku-ku, Tokyo, Japan; <sup>4</sup>Kitasato Institute for Life Sciences, Kitasato University, Minato-ku, Tokyo, Japan and <sup>5</sup>KIIM Pharmaceutical Laboratories Inc, Iwaki, Fukushima, Japan

Correspondence: Professor H Tanaka, Faculty of Pharmacy and College of Science and Engineering, Iwaki Meisei University, 5-5-1 Chuodai-Iino, Iwaki, Fukushima 970-8551, Japan.

E-mail: tanakaha@msn.com

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segment includes a structural module for a sugar-binding pocket formed by an LD-QXW sequence. However, the sugar-binding specificities of proteins are different to each other. For example, ricin toxin B-chain (RTB) from *Ricinus communis*, containing two units of  $\beta$ -trefoil fold, recognizes galactose,<sup>18</sup> whereas the xylan-binding domain of  $\beta$ -xylanase from *Streptomyces olivaceoviridis* E-86, consisting of one unit of the  $\beta$ -trefoil, binds to xylose oligomer.<sup>19</sup> The three modules (segments) of AH form the HMTG-binding pockets. The three-dimensional structure of the modules reveals that the active residues of the segments 1, 2 and 3 are well fitted to each other in both positions and orientations.<sup>20</sup> These identical aspects mean that each module is equivalently able to bind to an HMTG. Consequently, such multivalent interaction between three modules and three HMTGs could enhance the affinity of AH to HIV gp120. Similar affinity enhancement caused by multivalent interaction between carbohydrate ligands and receptors is well established as the glycoside cluster effect of lectin<sup>21</sup> and is a critical factor of AH for specific and potent anti-HIV activity. Furthermore, previous research showed that AH potently binds to a glycoprotein having many HMTGs, such as gp120, whereas it possesses very weak affinity against thyroglobulin or RNase B, having few HMTGs.<sup>16</sup> This suggests that the HIV gp120-specific lectin AH is an excellent anti-HIV agent with no side effects. In fact, AH has no mitogenic activity<sup>14,15</sup> and does not induce cytokines/chemokines secretion in peripheral blood mononuclear cell culture.<sup>14</sup>

Here, we present more active AH derivatives, AH dimers, which have higher anti-syngium formation and anti-HIV activities than that of AH monomer. This research may identify an excellent candidate for prevention of HIV infection.

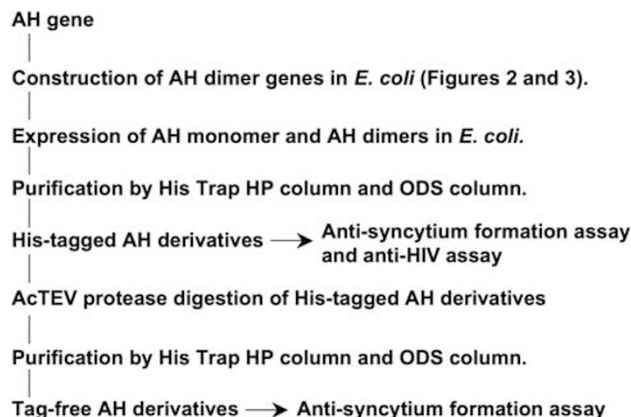
## MATERIALS AND METHODS

### Vectors, restriction enzymes, ligation and DNA preparation

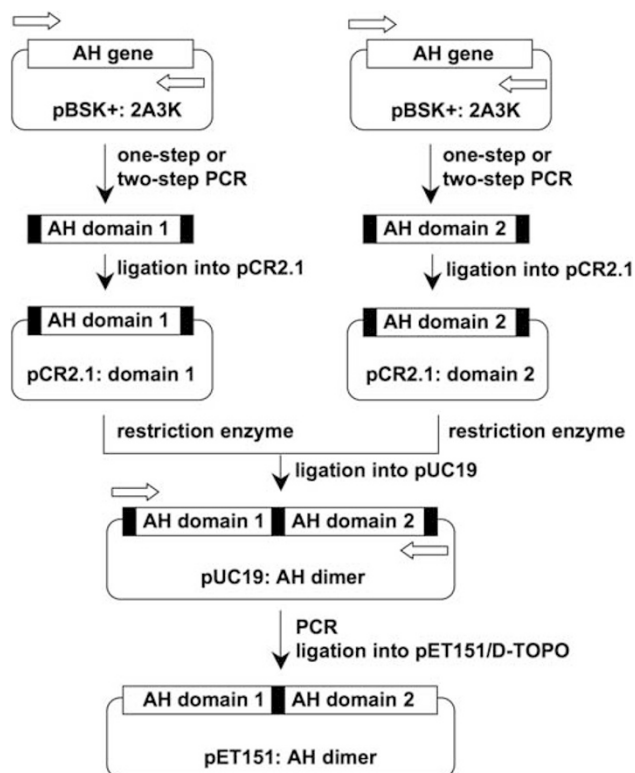
pBluescript II SK+: 2A3K (pBSK+:2A3K) containing the AH gene, was prepared as previously described.<sup>22</sup> Cloning vectors, pUC19 and pCR2.1 were purchased from Takara Bio (Shiga, Japan) and from Invitrogen (Carlsbad, CA, USA), respectively. An expression vector pET151/D-TOPO was obtained from Invitrogen. *Bam*H I, *Hind* III, *Eco*R I, *Nhe* I, *Mun* I and *Acc* III were purchased from Takara Bio. *Age*I was purchased from Promega (Madison, WI, USA). Each restriction enzyme was used according to the manufacturer's protocol. DNA ligation to cloning vectors was carried out using DNA Ligation Kit ver. 2.1 (Takara Bio) and ligation to an expression vector was performed according to the manufacturer's protocol. Plasmid DNA was amplified in *Escherichia coli* JM109 and prepared by QIAprep Spin Miniprep Kit (QIAGEN K K, Tokyo, Japan). DNA fragments derived from PCR products or restriction enzyme digestion were purified by agarose gel electrophoresis.

### Construction of AH dimer genes, Ala-substitution mutant genes and AH expression vector

Figure 1 shows a simple scheme of construction, purification and evaluation for biological activities of AH derivatives. Figure 2 shows a scheme of the construction of AH dimer genes. Each AH dimer contains two units of AH (domains 1 and 2, respectively, Figure 3b). The genes of domains 1 and 2 were generated by one or two-step PCR. Primer sets used are shown in Table 1. Amplified DNA fragments were ligated into pCR2.1. The genes of domains 1 and 2 were then digested with restriction enzymes and ligated into pUC19 to obtain plasmids containing full-length AH dimer genes. Using these plasmids as a template, and 151sen (C ACC GCC TCG GTG ACC ATC CGC) and 151asn (TTA TTA GCC GGT GTA CCA CTT CTG) as a primer set, PCR was run. Amplified products were ligated into pET151/D-TOPO to obtain AH dimer expression vectors. The Ala-substitution mutant genes of His-TEV-AH/RTB (132-143)/AH were prepared according to the scheme described in Figure 2, using pET30 Xa/LIC vector carrying AH (Y23A), AH (Y61A) or AH (Y99A) gene<sup>20</sup> as a template for AH domain 1 or AH domain 2, respectively. The AH monomer expression vector was also constructed through



**Figure 1** A simple scheme of construction, purification and evaluation for biological activity of actinohivin (AH) derivatives.

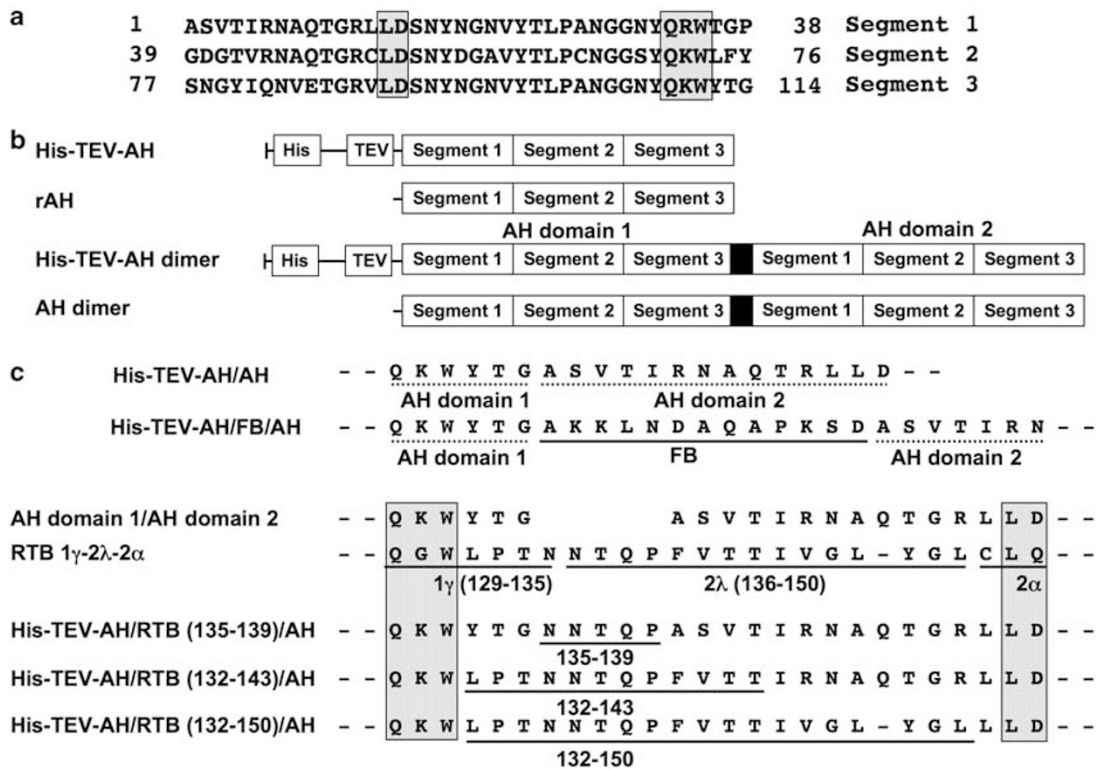


**Figure 2** A construction scheme of actinohivin (AH) dimer genes. White arrows indicate primers. Black boxes indicate restriction sites added to 5'/3' ends of each AH gene.

ligation into pET151/D-TOPO of the DNA fragment, which was generated by PCR using 151sen and 151asn as a primer set, with pBSK+: 2A3K as a template.

### Polymerase chain reaction (PCR)

For cloning to pCR2.1, PCR was carried out using a 0.2 mM deoxynucleotide triphosphate mixture, Takara Ex Taq buffer (Takara Bio), 5% dimethyl sulfoxide, 5 pmol of each primer and 0.5 U Takara Ex Taq DNA polymerase (Takara Bio) and 10 ng of pBSK+: 2A3K or 1 pmol of DNA fragment derived from first PCR. For cloning to pET151/D-TOPO, or for first PCR to obtain a template of second PCR, PCR was run using a 0.2 mM deoxynucleotide



**Figure 3** (a) The amino acid sequence of actinohivin (AH). Gray boxes indicate LD and QXW motifs, respectively. (b) A schematic diagram of AH monomer and AH dimer. His, 6×His (His tag); TEV, AcTEV protease recognition sequence; black boxes, a spacer region. (c) Spacer sequences used for AH dimerization. Underlined sequences: FB, corresponding to the residues 48–60 of fragment B of staphylococcal protein A; 1γ, 2λ and 2α, the sequences of sugar-binding pocket or spacer region of ricin toxin B-chain (RTB); 135–139, 132–143 and 132–150, the sequences derived from 1γ and 2λ segment of RTB. Gray boxes indicate LD and QXW motifs.

**Table 1** Primer sets used for construction of AH dimer genes

Gene (restriction sites) <sup>a</sup>	Primer sequences (5' → 3') of first PCR		Primer sequences (5' → 3') of second PCR	
	Sense	Anti-sense	Sense	Anti-sense
AH/RTB (135-139)/AH domain 1 ( <i>Bam</i> HI/ <i>Nhe</i> I)	GGATCCGCCTCGGTGACCATC	GCTAGCGGGCTGGGTGTT GTTG CCGGT GTA	—	—
AH/RTB (135-139)/AH domain 2 ( <i>Nhe</i> I/ <i>Hind</i> III)	GCTAGCGTGACCATCCGCAAC GCC	AAGCTTATTAGCCGGTGTACCAC	—	—
AH/RTB (132-143)/AH domain 1 ( <i>Bam</i> HI/ <i>Acc</i> III)	GGATCCGCCTCGGTGACCATC	GCTGGGTGTTGTTGGTGGGCAG CCACTTCT	GGATCCGCCTCGGTGACCATC	TCCGGATGGTGGTGACGAA GGGCTGGGTGT
AH/RTB (132-143)/AH domain 2 ( <i>Acc</i> III/ <i>Hind</i> III)	ATCCGGAACGCCAGACCGGC	AAGCTTATTAGCCGGTGTACCAC	—	—
AH/RTB (132-150)/AH domain 1 ( <i>Bam</i> HI/ <i>Mun</i> I)	GGATCCGCCTCGGTGACCATC	CTGGGTGTTGTTGGTGGGCAGC CACTTCTG	GGATCCGCCTCGGTGACCATC	GACAATTGTGGTGACGAAG GGCTGGGTGTT
AH/RTB (132-150)/AH domain 2 ( <i>Mun</i> I/ <i>Hind</i> III)	ATTGTCGGCCTGTACGGCCTC CTGCTGGAC	AAGCTTATTAGCCGGTGTACCAC	ACCCAGCCCTTCGTACCACA ATTGTCGGC	AAGCTTATTAGCCGGTGTAC CAC
AH/AH domain 1 ( <i>Bam</i> HI/ <i>Age</i> I)	GGATCCGCCTCGGTGACCATC	ACCGGTGTACCACTTCTGGTA	—	—
AH/AH domain 2 ( <i>Age</i> I/ <i>Hind</i> III)	ACCGGTGCCTCGGTGACCATC	AAGCTTATTAGCCGGTGTACCAC	—	—
AH/FB/AH domain 1 ( <i>Bam</i> HI/ <i>Hind</i> III)	GGATCCGCCTCGGTGACCATC	AAGCTTCTTGGCCTGGTGTGTA	—	—
AH/FB/AH domain 2 ( <i>Hind</i> III/ <i>Eco</i> R I)	GCGCCGAAGAGTGATGCCTCG GTGACC	GAATTCTTATTAGCCGGTGTAC CAC	AAGCTTAACGACGCTCAGGCG CCGAAG	GAATTCTTATTAGCCGGTGTAC CCAC

<sup>a</sup>Restriction sites added to 5'/3' ends of each domain gene.

triphosphate mixture, Takara LA Taq buffer (Takara Bio), 0.25 mM MgCl<sub>2</sub>, 5% dimethyl sulfoxide, 5 pmol of each primer and 0.5 U Takara LA Taq DNA polymerase (Takara Bio) and 10 ng of pUC19: AH dimer or pBSK+: 2A3K. The reaction was run for 30 cycles that consisted of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s and polymerization at 72 °C for 1 min for each cycle.

### Production and purification of AH derivatives

Native AH was obtained as previously described.<sup>12</sup> The pET151/D-TOPO carrying AH derivative gene was transferred into *Escherichia coli* BL21 (DE3) pLysS. The recombinant cells were cultured in Luria-Bertani (LB) medium containing 30 µg ml<sup>-1</sup> of ampicillin, 34 µg ml<sup>-1</sup> chloramphenicol and 2% glucose at 37 °C. The overnight culture was inoculated to final concentration of 1% in fresh LB medium containing the above antibiotics and then cultured at 37 °C. When the culture reached the appropriate density (OD<sub>600</sub>=0.3), isopropyl-thio-β-D-galactopyranoside was added to a final concentration of 1 mM to induce the expression of a fusion AH derivative, carrying the His-Tag. The cells were cultured for a further 2 h and collected by centrifugation at 8000×g for 15 min. The precipitated cells were washed with phosphate-buffered saline and suspended with binding buffer (5 mM imidazole, 0.5 M NaCl and 125 mM Tris-HCl, pH 8.0). After disruption by sonic treatment in an ice bath, the cell lysate was centrifuged at 10 000×g for 15 min to give an insoluble fraction containing a fusion AH derivative. The insoluble fraction was dissolved with denaturing buffer (binding buffer containing 6 M guanidine HCl) and loaded on a column of His Trap HP (GE Healthcare UK, Buckinghamshire, UK) previously equilibrated with denaturing buffer. The column was washed with a five-column volume of the same buffer and a fusion AH was eluted by a three-column volume of denaturing buffer containing 300 mM imidazole. Finally, His-tagged AH derivatives were purified by HPLC using an ODS column (Senshu Pak Pegasil ODS, 6×250 mm, Senshu Scientific, Tokyo, Japan) under the same conditions as previously described.<sup>22</sup> Fusion AHs were digested by the AcTEV protease (Invitrogen) according to the manufacturer's protocol. After digestion, guanidine-HCl was added to the reaction mixture at a final concentration of 6 M and the solution was loaded into a His Trap HP (GE Healthcare UK) column. The tag-free AH derivatives contained in the flow-through fraction and the wash fraction of His Trap HP column (GE Healthcare UK) were purified by ODS column (Senshu Scientific). Fusion AHs and tag-free AHs thus purified were dissolved in adequate amounts of sterilized water and used for experiments.

### Protein determination

The protein concentration was determined by BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). Bovine serum albumin was used as the standard.

### SDS-polyacrylamide gel electrophoresis

All AH derivatives were analyzed by SDS-12.5% polyacrylamide gel electrophoresis conducted in a tris-glycine buffer system. Each sample (3 µg per lane) was mixed with SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2.5% SDS, 20% glycerol, 0.01% bromophenol blue, 2.5 mM EDTA and 200 mM dithiothreitol) and heated at 95 °C for 5 min. The gel was stained using 0.05% Coomassie brilliant blue G-250.

### Evaluation of syncytium formation inhibitory activity

Anti-syncytium formation activity was measured using HeLa/T-env/Tat and HeLa/CD4/Lac-Z cells.<sup>23</sup> These cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (InterGen, Burlington, MA, USA), 0.1% NaHCO<sub>3</sub> and 100 µg ml<sup>-1</sup> kanamycin sulfate (Meiji Seika, Tokyo, Japan). Cells at a concentration of 8×10<sup>3</sup> cells per 50 µl were co-cultivated with 10 µl of a test compound in 96-well plates under 5% CO<sub>2</sub> at 37 °C for 24 h. The β-galactosidase activity in the cells was measured by *O*-nitrophenyl-β-D-galactopyranoside as a substrate. AH derivatives were dissolved in sterilized water and serially diluted with sterilized water to the desired concentrations.

### Anti-HIV assay

Anti-HIV assay was performed using two laboratory strains (NL4-3 and Bal) and seven clinically isolated strains of HIV-1 (36, 158, 182, 214, 242, 251 and

307), which were collected at the AIDS Clinical Center, International Medical Center of Japan and the multinuclear activation of β-galactosidase indicator cells.<sup>24</sup> Multinuclear activation of β-galactosidase indicator cells were cultured in a 96-well plate in the presence of various concentrations of AH derivatives. After 24 h of incubation at 37 °C, the culture media were replaced with fresh media containing virus and various concentrations of AHs. After 2 days of incubation, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactose. Anti-HIV activities of AH derivatives were measured by counting the number of blue-stained cells (virus infected cells) microscopically.

## RESULTS

### Reconstruction of AH expression vector

As described previously,<sup>22</sup> recombinant AH, which was prepared by expression of His-AH using pET30 Xa/LIC as an expression vector in *Escherichia coli* and then digested with a protease factor Xa, has comparable anti-syncytium formation activity to that of native AH produced by *Longispora albida* K97-0003T. On the other hand, His-AH containing 47 amino acids at the N-terminus of AH has significantly low inhibitory activity (IC<sub>50</sub>=500 nM.<sup>22</sup>) In contrast, as shown in Table 2, His-TEV-AH generated by pET151/D-TOPO, containing additional 36 amino acid residues, exhibited a similar activity to those of native AH and rAH generated from AcTEV protease digestion of His-TEV-AH. Therefore, pET151/D-TOPO was used as a new expression vector for preparation of His-TEV-AH derivatives.

### Design of AH dimers

The construction of AH dimers was performed by a head-to-tail fusion of two AH molecules (Figure 3b). His-TEV-AH/AH consists of two AH molecules fused directly, whereas other AH dimers contain a spacer peptide to avoid steric hindrance that may occur by direct fusion of two AH molecules, to facilitate their suitable orientation for efficient binding to HMTGs or to add flexibility for AH dimers (Figure 3c). His-TEV-AH/FB/AH contains a spacer of 13 amino acid residues derived from the residues 48–60 of fragment B of staphylococcal protein A,<sup>25</sup> which has been used to provide flexibility for a fusion protein.<sup>26</sup>

The residues 135–139, 132–143 or 132–150 of RTB were also used as spacers. The primary sequence of RTB is divided into eight segments, 1λ (1–16), 1α (17–59), 1β (60–100), 1γ (103–135), 2λ (136–150), 2α (151–183), 2β (187–226) and 2γ (228–262) and RTB contains two units of β-trefoil fold that consist of 1α, 1β and 1γ, and 2α, 2β and 2γ, respectively.<sup>27</sup> The 2λ (136–150) has a role in connection of two β-trefoil folds. Therefore, the use of 2λ sequence as

**Table 2** Anti-syncytium formation activities of AH monomer and AH dimers

Protein	IC <sub>50</sub> (nM) <sup>a</sup>
Native AH	127 ± 7
His-TEV-AH	111 ± 4
rAH	114 ± 5
His-TEV-AH/AH	33 ± 3
His-TEV-AH/FB/AH	22 ± 1
His-TEV-AH/RTB (135–139)/AH	31 ± 3
His-TEV-AH/RTB (132–143)/AH	6 ± 2
His-TEV-AH/RTB (132–150)/AH	> 250
AH/RTB (132–143)/AH	14 ± 2

Abbreviation: AH, actinohivin.

<sup>a</sup>Average ± s.d. of three repeated experiments.

a spacer is expected to facilitate suitable arrangement of an AH dimer to avoid undesirable steric hindrance and/or to enable efficient binding to HMTGs. However, because sugar-binding pockets are formed by LD-QXW sequences, we considered that the residues 132–150 of RTB, which consists of four residues of 1 $\gamma$  and 2 $\lambda$ , form a spacer region for connecting two  $\beta$ -trefoil folds and we performed AH dimerization using a full length or a part of the sequence. In three AH dimers, His-TEV-AH/RTB(135–139)/AH, His-TEV-AH/RTB(132–143)/AH and His-TEV-AH/RTB(132–150)/AH, two AH molecules were linked by the residues 135–139, 132–143 and 132–150 of RTB, respectively (Figure 3c). Then, to set the length of amino acid sequence from the QXW motif of the AH domain 1 to LD motif of the AH domain 2 to that of RTB, some residues of AH domains 1 and 2 were substituted for the RTB sequence (Figure 3c). His-TEV-AH dimers thus designed were prepared by the *Escherichia coli* expression system as described in Materials and Methods section. Highly purified AH derivatives showed single bands on SDS-polyacrylamide gel electrophoresis (Figure 4).

### Biological activities of AH dimers

The highly purified AH derivatives were subjected to anti-syncytium formation assay. Although His-TEV-AH/RTB(132–150)/AH showed reduced activity, His-TEV-AH/AH, His-TEV-AH/FB/AH and His-TEV-AH/RTB(135–139)/AH exhibited 4–6-fold higher activity than that of AH or His-TEV-AH (Table 2). In particular, His-TEV-AH/RTB(132–143)/AH showed 20-fold higher activity than that of His-TEV-AH. However, AH/RTB(132–143)/AH obtained by AcTEV protease digestion of His-TEV-AH/RTB(132–143)/AH has somewhat lower activity than that of His-TEV-AH/RTB(132–143)/AH (Table 2). To confirm whether all six HMTG-binding pockets of His-TEV-AH/RTB(132–143)/AH actually participate in HMTG binding, the Tyr

residue, which was identified as an HMTG-binding residue,<sup>20</sup> was substituted to Ala in one segment among six segments of the dimer, as shown in Figure 5. The six Ala-substituted mutants were subjected to anti-syncytium formation assay. All mutants show decreased activity (Table 3), which may imply that all six segments of His-TEV-AH/RTB(132–143)/AH participate in binding to HMTGs of gp120.

Anti-HIV activities of His-TEV-AH and His-TEV-AH/RTB(132–143)/AH against various HIV strains, including laboratory strains and clinical isolates, were also evaluated (Table 4). The His-TEV-AH/RTB(132–143)/AH showed 2–30-fold higher anti-HIV activity than His-TEV-AH against laboratory and clinically isolated HIV strains, including those resistant to reverse transcriptase and protease inhibitors.

**Table 3** Anti-syncytium formation activities of Ala-substituted mutants of His-TEV-AH/RTB (132–143)/AH

Protein	IC <sub>50</sub> (nm) <sup>a</sup>
His-TEV-AH/RTB (132–143)/AH	6 ± 0.3
His-TEV-AH (Y23A)/RTB (132–143)/AH	203 ± 17
His-TEV-AH (Y61A)/RTB (132–143)/AH	93 ± 17
His-TEV-AH (Y99A)/RTB (132–143)/AH	12 ± 3
His-TEV-AH/RTB (132–143)/AH (Y142A)	23 ± 3
His-TEV-AH/RTB (132–143)/AH (Y180A)	13 ± 0.3
His-TEV-AH/RTB (132–143)/AH (Y218A)	13 ± 1

<sup>a</sup>Average ± s.d. of three repeated experiments.

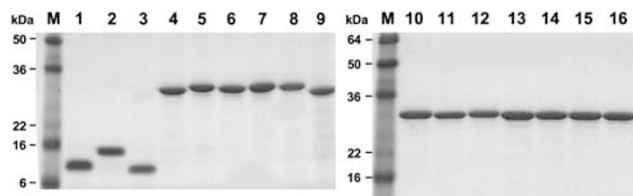
**Table 4** Anti-HIV activities of His-TEV-AH and His-TEV-AH/RTB (132–143)/AH

HIV strain	Drug-resistance <sup>a</sup>	IC <sub>50</sub> (nm)		
		His-TEV-AH	His-TEV-AH/RTB (132–143)/AH	Fold increase <sup>b</sup>
NL4-3	Sensitive	42	2.1	20.0
Bal	Sensitive	12	4.2	2.9
36	Sensitive	130	4.2	30.9
158	RTI/NNRTI/PI	22	2.1	11.0
182	NNRTI/PI	110	9.0	12.2
214	Sensitive	150	5.9	25.4
242	RTI/NNRTI/PI	190	76	2.5
251	RTI/PI	26	6.8	3.8
307	Sensitive	290	35	8.3

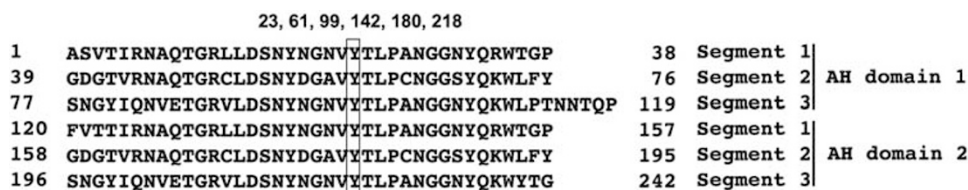
Abbreviations: RTI, reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

<sup>a</sup>Resistance to RTI, NNRTI and PI.

<sup>b</sup>Fold increase was calculated by dividing the IC<sub>50</sub>s of His-TEV-AH by those of His-TEV-AH/RTB (132–143)/AH.



**Figure 4** SDS-polyacrylamide gel electrophoresis of actinohivin (AH) derivatives. Lane 1, rAH; Lane 2, His-TEV-AH; Lane 3, native AH; Lane 4, His-TEV-AH/AH; Lane 5, His-TEV-AH/FB/AH; Lane 6, His-TEV-AH/RTB (135–139); Lane 7, His-TEV-AH/RTB (132–143)/AH; Lane 8, His-TEV-AH/RTB (132–150)/AH; Lane 9, AH/RTB (132–143)/AH; Lane 10, His-TEV-AH/RTB (132–143)/AH; Lane 11, His-TEV-AH (Y23A)/RTB (132–143)/AH; Lane 12, His-TEV-AH (Y61A)/RTB (132–143)/AH; Lane 13, His-TEV-AH (Y99A)/RTB (132–143)/AH; Lane 14, His-TEV-AH/RTB (132–143)/AH (Y142A); Lane 15, His-TEV-AH/RTB (132–143)/AH (Y180A); Lane 16, His-TEV-AH/RTB (132–143)/AH (Y218A); M, SeeBlue Plus2 Pre-Stained Standard (Invitrogen).



**Figure 5** The designed amino acid sequence of AH/RTB (132–143)/AH. The Tyr residue of each segment necessary for high mannose-type glycans (HMTG) binding was substituted to Ala in mutational analysis experiments.

## DISCUSSION

The actinomycete-derived lectin AH has potent anti-HIV activity against not only laboratory strains, but also clinically isolated strains.<sup>12</sup> AH consists of three HMTG-binding pockets,<sup>22</sup> which have high homology in the amino acid sequence of their LD-QXW motifs and this enables the three HMTG-binding pockets to bind cooperatively, specifically to three HMTGs on a gp120. Our previous report<sup>28</sup> indicated that, although AH mutants with only one or two HMTG-binding pocket(s) have no or very weak anti-synctium formation activity, respectively, native AH with three pockets has a strong activity, due to the so-called 'cluster effect' of lectin.<sup>21</sup> This suggests that further increase in the number of HMTG-binding pockets might enhance anti-synctium formation and the anti-HIV activities of AH. Therefore, we tried dimerization of AH to improve anti-HIV activity.

RTB belonging to CBM 13 possesses two units of  $\beta$ -trefoil fold, and the residues 136–150 (2 $\lambda$  segment) of RTB is defined as a spacer region to connect the two  $\beta$ -trefoil folds.<sup>27</sup> In the present study, this type of linkage was introduced between two AH molecules like RTB. The three AH dimers constructed, namely His-TEV-AH/RTB(135–139)/AH, His-TEV-AH/RTB(132–143)/AH and His-TEV-AH/RTB(132–150)/AH show different anti-synctium formation activities. This suggests that, in addition to the length of the spacers, their sequence compositions are important in AH dimers to ensure efficient HMTG binding. Comparison in the activities given in Table 2 suggests that it is possible to optimize the sequence composition of the residues 132–143 of RTB to obtain further improvement in the anti-HIV activity of an AH dimer.

To evaluate the HMTG binding at an individual pocket, six kinds of Ala-substituted derivatives mutated in each pocket were subjected to anti-synctium formation assay. As a result, the decreased anti-synctium formation activities shown in Table 3 may imply that all pockets of His-TEV-AH/RTB(132–143)/AH contribute to HMTG binding. However, the different extent of decreased activities (1/2–1/34) among the mutants suggests that the contributions of the six HMTG-binding pockets are not equivalent to each other, whereas three HMTG-binding pockets of AH monomer have a similar extent of HMTG-binding ability.<sup>28</sup> It seems that the segments 1 and 2 of AH domain 1 have a more important role in HMTG binding than segment 3 of AH domain 1 and three segments of AH domain 2. However, because these results were obtained in synctium formation assay, further experiments using various HIV strains are required to clarify this. Anyway, the improvement of the AH dimer's activity is considered to be brought about by increase in the number of HMTG-binding pockets. Balzarini<sup>29</sup> suggests that carbohydrate-targeting lectin has a new therapeutic concept. The long-term treatment of HIV by a carbohydrate-binding lectin will induce emergence of drug-resistant strains with deletion of N-linked glycans of gp120. Because dense N-linked glycan of gp120 has a role in a shield for hiding its immunogenic and antigenic epitopes,<sup>7</sup> HIV having a deglycosylated envelop may become prone to neutralization and elimination by the immune system. Therefore, exposure of HIV to an AH dimer with six HMTG-binding pockets is expected to generate much-deglycosylated virus more sensitive to the immune system rather than the one induced by AH monomer treatment. Thus, AH dimers have significant promise, meriting further investigation as a means to develop new anti-HIV therapy.

Anti-HIV assay of His-TEV-AH exhibited diverse sensitivities ( $IC_{50}$ =12–290 nM) to various HIV strains, including clinically isolated drug-resistant ones (Table 4). This might depend on the number of N-glycosylation sites on gp120, in particular on its C2 region and/or V4 region(s) of gp120.<sup>15</sup> Although it is unclear how many N-linked

glycans the strains tested in this paper possess on the gp120 molecules, strains less sensitive to AH monomer are speculated to have relatively few N-linked glycans. On the other hand, it is noteworthy that His-TEV-AH/RTB(132–143)/AH exhibited much higher activity (2–30.9 fold) than those of the AH monomer, especially potent anti-HIV activity, even against some strains (the 36, 182 and 214 strains) that have low sensitivities to AH monomer. The number of HMTG-binding pockets of His-TEV-AH/RTB(132–143)/AH participating in binding to HMTGs of each HIV strain remains to be clarified. However, the improved activity is considered to be brought about by an increase in the number of HMTG-binding pockets. Thus, in addition to improving anti-HIV activity, dimerization of AH also produces a broader anti-HIV spectrum against various HIV strains, including low sensitive ones to AH monomer. Further studies are needed to clarify the relationship between the numbers (and/or location) of HMTG of gp120 and anti-HIV activity of AH; for example, genotypic characterization of gp120 of HIV-1 strains tested.

Recently, the Center for the AIDS Program of Research in South Africa 004 trial<sup>30</sup> assessed the effectiveness and safety of a gel formulation of tenofovir, a nucleotide reverse transcriptase inhibitor, for prevention of HIV infection in women. Because tenofovir-resistant strains are considered to occur, combination of AH dimer with tenofovir is expected to improve prevention of transmission of HIV-1 strains, including drug-resistant ones, although animal testing using macaques will be necessary before human trials can begin.

In conclusion, we have demonstrated that: (1) dimerization of AH improves its anti-HIV activity and broadens the spectrum of higher anti-HIV activity; (2) the diversity of anti-synctium formation activity of AH dimers with different spacer sequence compositions indicates the possibility that AH dimer derivatives having much higher anti-HIV activity are created by optimization of a spacer sequence; (3) AH dimers, effective even against drug-resistant strains, represent good candidates for use in developing a useful HIV-1 microbicide targeting virus envelop glycan.

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