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Actinohivin, a novel anti-human immunodeficiency virus protein from an actinomycete, inhibits viral entry to cells by binding high-mannose type sugar chains of gp120

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Abstract

We searched human immunodeficiency virus (HIV) entry inhibitors and found a novel anti-HIV protein, actinohivin (AH), in a culture filtrate of the newly discovered genus actinomycete *Longispora albida* gen. nov., sp. nov. This paper deals with the mechanism of action of the anti-HIV activity of AH. AH exhibited potent anti-HIV activities against various strains of HIV-1 and HIV-2. AH bound to the glycoprotein gp120 of various strains of HIV-1 and gp130 of simian immunodeficiency virus (SIV), but did not bind to non-glycosylated gp120 nor to cells having CD4 and coreceptors, suggesting that AH inhibits viral entry to cells by binding to the envelope glycoprotein. The investigation of the effects of various sugars on AH–gp120 binding by ELISA revealed that yeast mannan alone strongly inhibited the binding ($IC_{50} = 3.0\,\mu g/ml$). Experiments investigating the binding of AH to other glycoproteins revealed that AH binds to ribonuclease B and thyroglobulin that have a high-mannose type saccharide chain, but not to other glycoproteins having a *N*-glycoside type saccharide chain. The above results indicate that high-mannose type saccharide chains of gp120 are molecular targets of AH in its anti-HIV activity.

Infection of cells with human immunodeficiency virus type-1 (HIV-1) requires fusion of the cellular and viral membranes, which is mediated by viral envelope glycoproteins (gp120 and gp41) and cell surface receptors (CD4 and a chemokine receptor such as CCR5 or CXCR4) on the target cells [1–4]. This step is an attractive target for selective anti-HIV-1 therapy. CD4-positive ($CD4^+$) cells infected with HIV-1 contain the viral envelope glycoprotein complex gp120/gp41 on their surface and merge with healthy $CD4^+$ cells to produce multinuclear giant cells (syncytia) [5,6]. Syncytium formation is an indirect but major mechanism of death of $CD4^+$ cells in HIV infection. The cell–cell fusion mimics infection of cells with HIV-1. Based on these findings, we established cell lines that stably express the HIV envelope glycoprotein complex and constructed a syncytium formation assay system without handling intact HIV-1 virus [7].

During our screening program for anti-HIV agents having anti-syncytium formation activity, a new anti-HIV protein, designated as actinohivin (AH), was isolated from a cultured broth of actinomycete strain K97-0003, which was classified as a new genus actinomycete *Longispora albida* gen. nov., sp. nov. [8,9]. AH had a unique sequence consisting of 114 amino acids and highly conserved internal sequence triplication (comprising amino acids 1–38, 39–77, and 78–114; segments 1, 2, and 3, respectively) [10]. AH potently inhibited syncytium formation and cytopathicity caused by $HIV\text{-}1_{IIIB}$ infection.

In this paper, we present evidence that AH inhibits the infection of both T- and M-tropic HIV-1 strains in the susceptive cells and that AH binds selectively to high-mannose type sugar chains of gp120 but not to CD4 and coreceptor-expressing cells.

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Materials and methods

Cells. HeLa/T-env/Tat, HeLa/CD4/LacZ, HeLa/M-env/Tat, and HOS/CD4/CCR5/LacZ cell lines were established as described previously [7]. HOS/CD4/CXCR4 cells were obtained from the AIDS Research and Reference Program (NIAID, NIH). All the above cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) and 100 μg/ml kanamycin sulfate (Meiji Seika, Tokyo, Japan).

Reporter gene activation-mediated syncytium formation assay. The assay was performed as described previously [7]. HeLa/CD4/LacZ or HOS/CD4/CCR5/LacZ cells, a sample solution, and then HeLa/T-env/Tat or HeLa/M-env/Tat cells were put in each well of a 96-well microplate. They were cocultured under 5% CO_2 at 37 °C overnight. After the culture media were removed, the cells were lysed with 20 μl of 0.05% Tween 20. The lysate was mixed with 80 μl of a buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol) and 20 μl of 0.5 mg/ml *O*-nitrophenyl-β-D-galactopyranoside and incubated for 80 min at 37 °C. The reaction was stopped with 50 μl of 2 M Na_2CO_3, and the absorbance at 405 nm was measured with a microplate reader.

Anti-HIV assay. Four strains of HIV-1 (HTLV-IIIB, O18A, NL4-3, and JR-FS) and two strains of HIV-2 (ROD and EHO) were used for the anti-HIV assay. The activities of AH against these viruses were measured based on the inhibition of virus-induced infectious focus formation in the multinuclear activation of galactosidase indicator (MAGI) cells [11]. MAGI cells were cultured in a 96-well plate in the presence of various concentrations of AH. After 24 h of incubation at 37 °C, the culture media were replaced with fresh culture media containing virus and various concentrations of AH. After 2 days of incubation, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactose. The number of infected blue cells was counted microscopically.

Preparation of anti-AH polyclonal antibody. A New Zealand white rabbit was initially immunized with 500 μg AH in Freund's complete adjuvant. Two follow-up booster injections of 300 μg AH in Freund's incomplete adjuvant were administered at 30-day intervals. The immunoglobulin fraction of the resultant immune serum of rabbit was isolated by protein A affinity chromatography (Sepharose HiTrap rProtein A, Amersham Biosciences, Piscataway, NJ).

ELISA studies of AH interactions with viral envelope glycoproteins and sCD4. To determine the affinities of AH to envelope glycoproteins and recombinant soluble CD4 (sCD4, Genentech, San Francisco, CA), 100 ng/well of the following proteins was bound to a 96-well protein adsorbing plate: $gp120_{SF2}$ (recombinant wild type from HIV-1_{SF2} expressed in CHO cells), $gp120_{MN}$ (recombinant from HIV-1_{MN} expressed in baculovirus), gp130 (from $SIV_{mac}239$ expressed in CHO cells), and non-glycosylated gp120 (recombinant from HIV-1_{SF2} gp120 expressed yeast cells), all obtained from the AIDS Research and Reference Program (NIAID, NIH); $gp120_{IIIB}$ (Genentech); and bovine serum albumin (BSA). The plate was blocked with non-fat dried milk, washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and incubated for 2 h with various concentrations of AH and then washed four times with PBS-T. The bound AH was determined by incubation first with a solution of anti-AH rabbit polyclonal antibody preparation (1:2000), washed four times with PBS-T, and then incubated with sheep–anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Bioscience). After washing four times with PBS-T, the plate was incubated with *o*-phenylenediamine dihydrochloride (OPD) solution (0.45 mg/ml OPD, 16 mM citrate, and 50 mM disodium hydrogenphosphate, pH 5.0) for 8 min and then quenched by 2 N H_2SO_4. Antibody binding was measured by determining absorbance at 405 nm.

Binding of [^{125}I]SDF-1 to HOS/CD4/CXCR4 cells. After HOS/CD4/CXCR4 cells (2.5×10^4 cells/well) were cultured in a 48-well plate (Asahi Techno Glass, Chiba, Japan) for 24 h, the culture media were replaced with the binding buffer (DMEM containing 20 mM Hepes and 0.5% BSA, pH 7.2). Binding reaction was performed at room temperature for 40 min in the presence of 0.1 nM [^{125}I]stromal derived factor-1 alpha (SDF-1) (specific activity 2000 Ci/mmol, Amersham Biosciences) and various concentrations of AH. The binding reaction was terminated by washing out the free ligand with cold PBS, and the cell-associated radioactivity was counted by a liquid-scintillation counter (Beckman Instruments, Fullerton, CA).

Binding interaction of AH- and env-expressing cells. HeLa/T-env/Tat or HeLa/CD4 cells (1×10^6 cells) were cultured in a 60-mm diameter dish (Asahi Techno Glass) overnight. After the cells were washed with PBS, media containing various concentrations of AH were added to the cells and incubated for 1 h at 37 °C. After washing four times with PBS, the cells were collected with a policeman and centrifuged at 800*g* for 5 min at 4 °C. After removal of the supernatant, the cell pellets were resuspended with lysis buffer (1% NP-40, 0.5% deoxycholic acid, 150 mM NaCl, and 50 mM Tris–HCl, pH 8.0) and placed on ice for 30 min. After centrifugation at 9000*g* for 5 min at 4 °C, supernatant was subjected to SDS–15% polyacrylamide gel electrophoresis. Proteins in polyacrylamide gel were electrophoretically transferred to a membrane (Immobilon, Millipore, Bedford, MA) using a semi-dry blotting apparatus (Biocraft, Tokyo, Japan). The membrane was immersed with a 3% solution of non-fat dried milk in PBS containing 0.5% Tween 20 (PBS-T) overnight at 4 °C. The membrane was then washed three times with PBS-T. AH was detected by addition of a rabbit anti-AH polyclonal antibody preparation (1:1000 dilution), followed by alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G preparation (1:2000 dilution). The membrane was then washed three times with PBS-T and rinsed in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5). The membrane was developed with alkaline phosphate buffer containing 337.5 μg/ml nitroblue tetrazolium chloride (Roche Diagnostics, Indianapolis, IN) and 175 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (Roche Diagnostics).

Effects of sugar on AH–gp120 interaction. We used the ELISA method to search for substances that affect AH–gp120 interaction. Each well of a 96-well protein adsorbing plate was coated with 2 μg/ml AH and then blocked with a 5% solution of non-fat dried milk at room temperature for 1 h. After washing four times with PBS-T, a solution (50 μl) containing 0.18 μg/ml $gp120_{IIIB}$ and various concentrations of tested EDTA, salts, detergents, or sugars (glucose, galactose, fructose, fucose, rhamnose, mannose, arabinose, xylose, *N*-acetylglucosamine, *N*-acetylgalactosamine, methyl-α-mannopyranoside, xylan, inulin, paramylon, pullulan, dextran sulfate (MW 5000), chitosan, levan, coffee mannan, and yeast mannan) was added to wells and incubated for 2 h. The plates were then washed four times with PBS-T, and the bound gp120 was determined by 0.3 μg/ml anti-gp120 mouse monoclonal antibody 5B3 (Genentech).

Hemagglutination assay. Hemagglutination was carried out at 37 °C using rabbit, sheep erythrocytes (Cosmo Bio, Tokyo, Japan) and human blood. AH and concanavalin A (Sigma Chemical, St. Louis, MO) were diluted with PBS in microtiter plates (25 μl/well) and erythrocyte suspension (25 μl of 2% v/v) was then added to the plates. The results were noted after 2 h.

Results

Effect of AH on syncytium formation

The effect of AH on syncytium formation was examined using previously established recombinant cells [7]. When the combinations of HeLa/T-env/Tat with HeLa/CD4/LacZ cells (T-tropic system) and HeLa/M-env/Tat

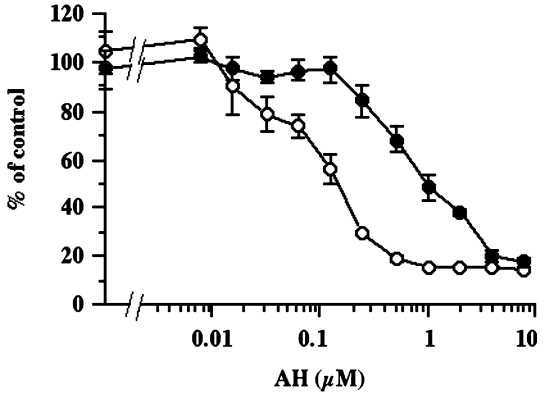


Fig. 1. Inhibitory effects of AH on syncytium formation in a coculture of HeLa/T-env/Tat and HeLa/CD4/LacZ cells (T-tropic system, open circle), HeLa/M-env/Tat and HOS/CD4/CCR5/LacZ cells (M-tropic system, closed circle). The inhibition of syncytium formation is displayed as percent against β-galactosidase activity of drug-free control cells. Error bars indicate standard deviation of the mean values obtained from triplicate samples.

with HOS/CD4/CCR5/LacZ cells (M-tropic system) were used, AH inhibited both T- and M-tropic syncytium formation as shown in Fig. 1. The IC_{50} values were 60 and 700 nM, respectively.

Anti-viral activities of AH against various strain of HIV

As described previously [8], AH potently inhibits HIV-1_{IIIB}-induced cytopathic effect in MT-4 cells ($EC_{50} = 230$ nM), but it exhibits no cytotoxicity even at the concentration of 100 μM used in HeLa cells with the MTT method. We examined anti-HIV activity of AH against various HIV strains. Table 1 shows that AH had potent inhibitory activities against T-tropic HIV-1 strains, IIIB, NL4-3, and O18A (primary isolate) at 2–110 nM and M-tropic HIV-1 strains and JR-CFS at 38 nM, indicating that AH inhibited the infection of both T- and M-tropic HIV-1. Furthermore, AH inhibited the infection of cells with HIV-2. Thus, it appears that AH recognizes a common target in these viruses and inhibits the entry to the susceptible cells.

Table 1
Anti-retroviral activity of AH measured by MAGI assay[a]

Virus strain	IC_{50}[b] (nM)
T-tropic HIV-1	
IIIB	2
O18A(primary isolate)	110
NL4-3	16
M-tropic HIV-1	
JR-CFS	38
HIV-2	
ROD	14
EHO	3.7

[a] The assay was done as described in the text.
[b] The IC_{50} values were calculated on the basis of reduction of blue cells induced by viral infection.

Interaction of AH with glycoproteins of immunodeficiency viruses

The interaction of AH with HIV-1 entry-related molecules, gp120, CD4, and chemokine receptors, was examined by ELISA using rabbit polyclonal anti-AH antibody for the detection of bound AH. First, the direct interaction of AH with gp120, sCD4, and bovine serum albumin (BSA, negative control) was examined. AH specifically bound to gp120 in a concentration-dependent manner, but hardly bound to sCD4 and BSA (Fig. 2A). Additional evidence of the specificity of the association of AH with gp120s of other strains of HIV-1 and with analogous SIV glycoprotein was demonstrated

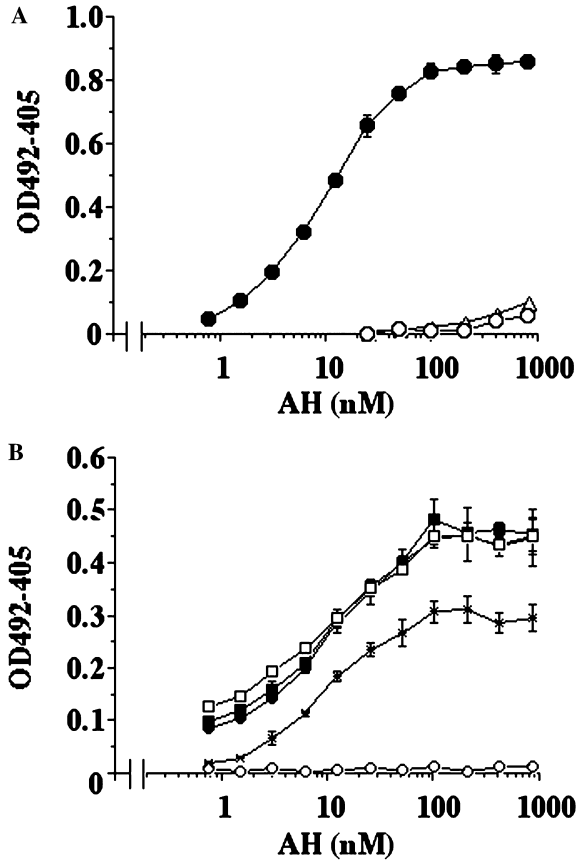


Fig. 2. ELISA study of the binding of AH to gp120. Proteins (100 ng/well) were bound to a 96-well plate and the plate was incubated with various concentrations of AH. Bound AH was visualized with anti-AH polyclonal antibodies as indicated by absorbance at 405 nm. Error bars are standard deviations based on at least triplicate determinations. (A) $gp120_{IIIB}$ (closed circle), rsCD4 (open triangle), and BSA (open circle). (B) gp120:HIV-1_{IIIB} (closed circle), HIV-1_{SF2} (closed square), HIV-1_{MN} (open square), $SIV_{mac}239$ (cross), and non-glycosylated HIV-1_{SF2} env (open circle).

in a similar ELISA format (Fig. 2B). The non-glycosylated HIV-1_{SF2} gp120 showed no binding to AH although glycosylated gp120s and $SIV_{mac}239$ gp130 were bound dose-dependently to AH. In addition, AH did not bind to HIV-1 gp41 peptide (env aa466–753) prepared from gp160 (data not shown). The above data indicate that AH binds to gp120 of both T- and M-tropic HIV-1 strains (HIV-1_{SF2} and HIV-1_{MN}, respectively) and to gp130 of SIV, and suggest that it binds to a sugar chain of the glycoproteins. Thus, AH interferes with viral infection regardless of cell tropism of HIV-1.

AH binds to env-expressing cells but not to receptor-expressing cells

The interaction of AH with env glycoproteins and other cell components expressed on cells was examined by Western blot analysis using polyclonal anti-AH antibody. The band of AH that was bound to cells and then detected from cells by the treatment with lysis buffer was detected with env-expressing cells (HeLa/T-env), but no bands were detected with non-env-expressing cells (HeLa/CD4) (Fig. 3). The band of AH was also detected with HeLa/M-env, but not with HeLa, HOS/CD4/CCR5, HOS/CD4/CXCR4, or NIH/3T3 cells (data not shown). Furthermore, AH did not inhibit the cell–cell fusion between ICAM-I-expressing HL60 cells stimulated with lipopolysaccharide or tumor necrosis factor and fluorescent-labeled HL60 cells (data not shown). Thus, it was confirmed that AH binds to env expressed on cells but it does not bind to other cell components including HIV receptors expressed on cells.

No effect of AH on SDF-1-CXCR4 binding

The effect of AH on the binding of SDF-1 to chemokine receptor (CXCR4)-expressing cells (HOS/CD4/CXCR4) was examined. An anti-HIV peptide, T-140, inhibited the binding with IC_{50} value of 1.47 μM, but AH did not inhibit it at all, even at the concentration of 8 μM (data not shown).

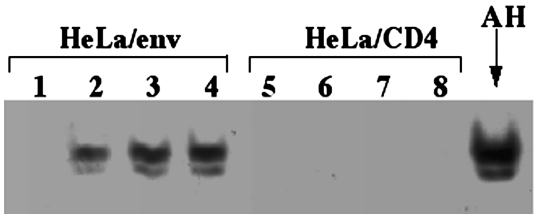


Fig. 3. Western blot analysis of the binding specificity of AH to cell surface envelope. For Western blotting, the membrane to which proteins in polyacrylamide gel were transferred was incubated with rabbit anti-AH polyclonal antibody, washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG. Lanes 1 and 5 were not treated. The other lanes were treated with 0.1 (lanes 2 and 6), 0.5 (lanes 3 and 7), or 1.0 μg/ml (lanes 4 and 8) of AH.

Substances that affect AH–gp120 binding

To identify the binding site of gp120 to AH, we studied substances that affect AH–gp120 binding. First, we examined the effect of EDTA, salts, and detergents, and found that they had no or only weak effects. For example, 10 mM NaCl or 1.0% Triton X-100 exhibited no effects, and high concentrations of EDTA (10 mM), ovalbumin (20 mg/ml), and BSA (20 mg/ml) had weak inhibitory effects (26.8%, 19.7%, and 11.7%, respectively).

Next, we examined the effects of various sugars on AH–gp120 binding because it was suggested from the above results that AH binds to the sugar chains of envelope glycoproteins. When $gp120_{IIIB}$ and various concentrations of a sugar were added simultaneously to an AH-coated plate and then incubated for 120 min at room temperature, all the monosaccharides and disaccharides tested exhibited IC_{50} values of more than 20 mg/ml (Table 2), although mannose and *N*-acetylglucosamine exhibited weak inhibitory effects on AH–gp120 binding (20% and 30%, respectively, at 10 mg/ml) (Fig. 4A). Among polysaccharides tested, xylan, inulin, paramylon, and pullulan had no effects even at 20 mg/ml, and dextran sulfate, chitosan, levan, and coffee mannan had weak effects (Fig. 4A and Table 2). On the other hand, yeast mannan exhibited a very strong effect ($IC_{50} = 3.0$ μg/ml) (Fig. 4A and Table 2). Then, we examined the effects of the above sugars on AH–gp120 binding when an AH-coated plate was treated with a sugar for 2 h before the addition of gp120 or 1 h after the addition of gp120. When the AH-coated plate was treated with a sugar before the addition of gp120, the effect of chitosan was somewhat decreased and that of

Table 2
Effects of various sugars on AH–gp120 binding

Sugars	IC_{50} (mg/ml)
D-Glucose	>20
D-Galactose	>20
D-Fructose	>20
L-Rhamnose	>20
D-Mannose	>20
D-Arabinose	>20
D-Xylose	>20
N-Acetyl-D-glucosamine	>20
N-Acetyl-D-galactosamine	>20
Methyl-α-mannopyranoside	>20
Sucrose	>20
Maltose	>20
Xylan	>20
Inulin	>20
Paramylon	>20
Pullulan	>20
Dextran sulfate (5000)	2.0
Chitosan	4.27
Levan	0.29
Coffee mannan (β-type)	0.6
Yeast mannan (α-type)	0.003

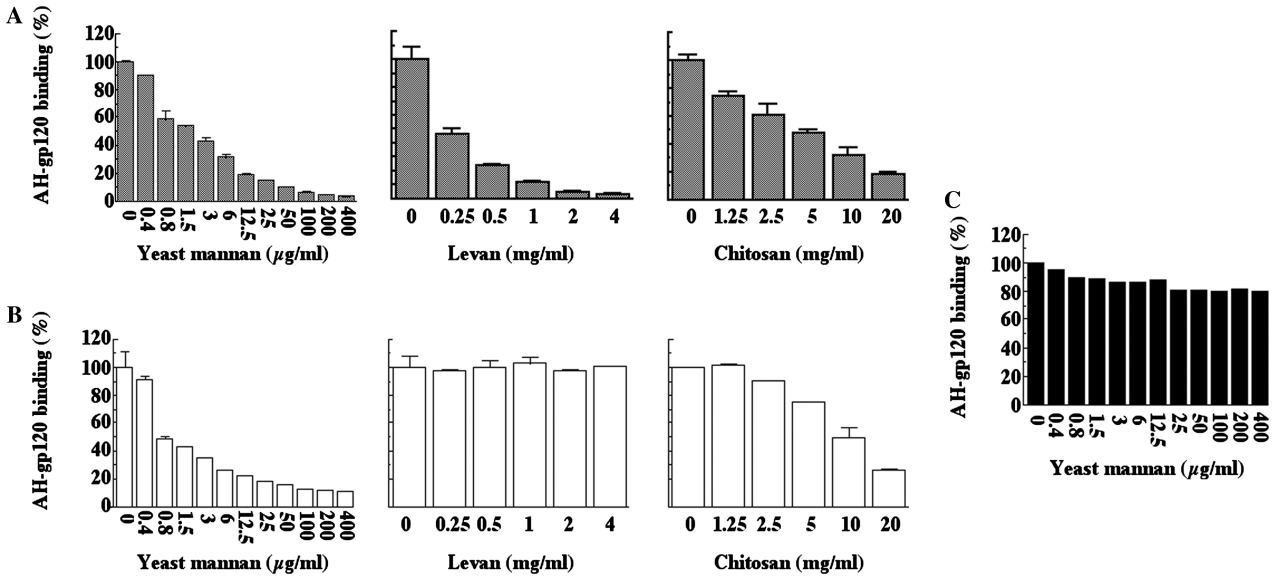


Fig. 4. Effects of sugars on AH–gp120 binding. (A) An AH-coated plate was incubated with a solution containing a sugar and $gp120_{IIIB}$ for 2 h. (B) After an AH-coated plate was incubated with a sugar solution for 1 h, the solution was removed and then the plate was incubated with a gp120 solution for 2 h. (C) After an AH-coated plate was incubated with a gp120 solution for 1 h, the solution was removed and then the plate was incubated with a yeast mannan solution for 2 h.

levan was vanished, while that of yeast mannan was somewhat increased (Fig. 4B). However, when an AH-coating plate was treated with yeast mannan after gp120 addition, the inhibiting effect of yeast mannan was not observed (Fig. 4C). The above data suggest that AH binds to gp120 sugar chains, especially high-mannose sugar chains.

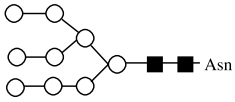
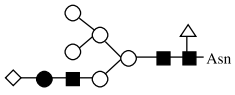
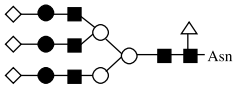
Hemagglutination properties

The fact that AH recognizes sugar chains of gp120 suggests that AH is a member of the lectin family. Lectin often causes cytotoxicity by binding to sugar chains on the cell surface. Therefore, we examined the hemagglutination (erythrocyte agglutination) activity of AH with erythrocytes from sheep, rabbits, and human. Concanavalin A and pea lectin were used as positive controls. The hemagglutination activity of concanavalin A with sheep erythrocytes was observed at all the concentrations tested (1.2–1250 μg/ml), but those of pea lectin and AH were not observed. With rabbit erythrocytes, concanavalin A and pea lectin exhibited hemagglutination activities at all the above concentrations; however, AH exhibited a very weak hemagglutination-like activity at concentrations higher than 150 μg/ml (12 μM). The concentration of AH that exhibited a hemagglutination activity is over 1000 times the concentration that exhibited anti-HIV activity. Thus, weak hemagglutination activity is not considered to relate directly to anti-HIV activity. With human erythrocytes, AH did not have any hemagglutination activity even at a concentration of 1250 μg/ml.

Binding specificity of AH to glycoproteins having a high-mannose sugar chain

To investigate the binding specificity of AH to glycoproteins, the binding activities of AH to glycoproteins other than HIV-1 gp120 were examined. The sugar chains of HIV-1 gp120 consist of N-linked glycans, but no O-linked glycans (mucin type). N-linked glycans are classified into high-mannose type including

Table 3
Subgroups of N-linked glycans and glycoproteins

N-linked glycans[a]	Glycoproteins
High-mannose type 	Ribonuclease B type III (bovine pancreas) Thyroglobulin (bovine) HIV-1 gp120
Hybrid type 	Ovalbumin (chicken) HIV-1 gp120
Complex type 	Fetuin (fetal bovine serum) Glycoprotein α-acid (human) Thyroglobulin (bovine) Transferrin (human) HIV-1 gp120

[a] Symbols: mannose, open circle; galactose, closed circle; *N*-acetylglucosamine, closed square; sialic acid, open diamond; fucose, open triangle.

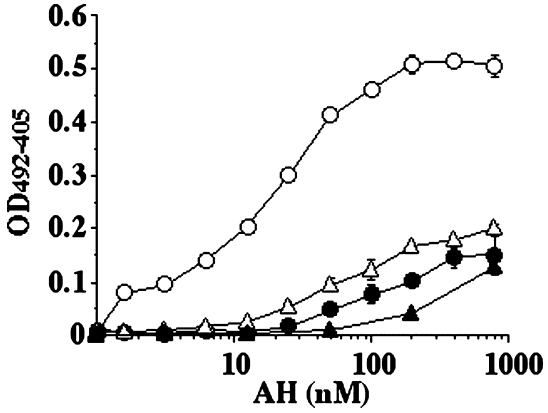


Fig. 5. Binding of AH to N-linked glycans containing a high-mannose type oligosaccharide. An ELISA plate was coated with HIV-1_{IIIB} gp120 (open circle, 100 ng/well), thyroglobulin (open triangle, 500 ng/well) or ribonuclease B (closed circle, 500 ng/well), ovalbumin (closed triangle, 500 ng/well).

N-acetylglucosamine, mannose, fucose, galactose, and *N*-acetylmuramic acid; complex type including mannose and *N*-acetylglucosamine; and hybrid type, a hybrid between high-mannose type and complex type (Table 3). HIV-1 gp120 contains all these types. The affinities of the glycoproteins shown in Table 3 to AH were assayed by ELISA using a glycoprotein-coated plate. AH was added to a glycoprotein-coated plate and then AH bound to the plate was detected with polyclonal anti-AH antibody. As a result, only high-mannose type glycoproteins (bovine ribonuclease B and bovine thyroglobulin) among the tested glycoproteins exhibited affinity to AH, although the affinity was much less than that to gp120 and ovalbumin (hybrid type) showed very much less affinity (Fig. 5). The binding of AH to ribonuclease B and thyroglobulin was also reduced by the addition of yeast mannan (data not shown), as observed with the binding of AH to gp120. AH binding to gp120, ribonuclease B, and thyroglobulin was observed by the lectin blotting method after separation by SDS–PAGE, although ovalbumin also exhibited a very weak affinity to AH (data not shown). The above results indicate that AH has an affinity to glycoproteins containing high-mannose type sugar chains but not to core sugar chains commonly contained in *N*-glycoside type glycoproteins.

Discussion

AH was discovered as a novel syncytium formation-inhibiting protein having anti-HIV activity from an actinomycete *L. albida* gen. nov., sp. nov. [8,9]. AH inhibits both T- and M-tropic syncytium formation as well as the infection of susceptible cells by various HIV strains, including T- and M-tropic HIV-1 and HIV. AH has no protease activity and no inhibitory activity against Sendai virus-induced cytopathicity [8].

In an ELISA experiment, we found that AH binds to the envelope glycoproteins gp120 of HIV-1 and gp130 of SIV, but not to non-glycosylated ones. Furthermore, AH binds to env-expressing cells but does not bind to cells having CD4 and a chemokine receptor. Thus, AH binds to sugar chains of virus surface env protein and inhibits infection. To search the binding target of AH, we tested the effects of various substances on AH–gp120 binding. The AH–gp120 binding was not inhibited by the presence of BSA, salts, EDTA, or detergents. We tested the effects of various sugars and found that AH–gp120 binding was not inhibited by the presence of high concentrations of monosaccharides and disaccharides. Among various sugars tested, yeast mannan (α-type) alone strongly inhibited the binding with an IC_{50} value of 3 μg/ml, unlike coffee mannan (β-type). Thus, the AH–gp120 interaction is different from those of classical carbohydrate-interactive lectins such as concanavalin A, jacalin, and *Galanthus nivalis* agglutinin, which are known to bind to gp120 in a monosaccharide-specific manner and to exhibit anti-HIV activities [12].

HIV-1 gp120 is extensively glycosylated with N-linked glycans for nearly half of its molecular mass [13]. All 24 potential carbohydrate attachment sites of HIV-1_{IIIB} strain gp120 produced in CHO cells are used for glycosylation, including 13 sites that contain complex type and 11 sites that contain either high-mannose type or hybrid type sugar chains [14]. We examined whether AH binds to other glycoproteins having these three types of N-linked glycans. Consequently, we found that AH binds to ribonuclease B and thyroglobulin having high-mannose type oligosaccharide, although these affinities are much less than that to gp120. The affinity to ovalbumin (hybrid type) was very much less than that to gp120. These data indicate that AH binds to high-mannose type sugar chains of gp120, thus inhibiting the entry of HIV-1 to susceptible cells. Gp120 has an unusually high number of high-mannose oligosaccharide chains compared with mammalian glycoproteins. Many of these glycosylation sites are conserved among HIV isolates, even though some are found in hypervariable regions of envelope glycoproteins. Eleven of the 24 sugar chains of gp120 are high-mannose or hybrid type [14]. Bovine thyroglobulin has four high-mannose sugar chains, which contain seven to nine mannose units, among 14 sugar chains [15]. Bovine ribonuclease B has only one high-mannose sugar chain, which contains seven or eight mannose units [16]. Ovalbumin has only one hybrid type sugar chain, which contains five or six mannose units [17]. The result that AH binds to high-mannose type sugar chains of gp120 correlates well with the finding that AH inhibits the entry of both T- and M-tropic HIV-1 and HIV-2. Thus, AH recognizes the common target point high-mannose sugar chains in HIV

env glycoprotein. AH appears to recognize α-linked di-, tri- or larger mannosides contained in both gp120 and yeast mannan (α-type), because gp120 does not contain large tracts of mannan-type structure [14]. Furthermore, the above results indicate that AH binds avidly to such oligosaccharide structures present on gp120 because its binding to gp120 is stronger than to glycoproteins bearing fewer glycosylation sites.

Bewley and Otero-Quintero [18] have shown that cyanovirin-N [19], an anti-HIV protein consisting of 101 amino acid residues, recognizes the terminal α(1–2) di-mannose structure of Man-8 and Man-9 glycans.

The C-type (calcium-dependent) lectin, dendritic cell (DC)-specific intracellular adhesion molecule (ICAM)-grabbing non-integrin (DC-SIGN) expressed on dendritic cells captures and transfers HIV and SIV efficiently to receptor-positive cells via the viral envelope glycoprotein [20,21]. DC-SIGN-positive cells are abundant in both human and rhesus macaque rectal and vaginal mucosa; therefore, DC-SIGN might play an important role in sexual and vertical transmission. Based on the crystal structure of the DC-SIGN carbohydrate recognition domain (CRD) and modeling of N-linked sugar chain, DC-SIGN binds to the outer tri-mannose branch point, which is present only in high-mannose type oligosaccharides [22]. Recently, Lin et al. [23] reported that, DC-SIGN selectively interacts with HIV env and Ebola virus glycoproteins containing more high-mannose than complex carbohydrate structures. Ebola virus glycoproteins are heavily glycosylated and contain both N-linked and O-linked glycans; it also has been reported that the infection of Zaire strain *Ebola* virus is inhibited by cyanovirin-N [24]. These studies and our results suggest that the carbohydrate moiety on viral surface proteins is a common anti-viral molecular entry target.

Our data show that AH binds to high-mannose type saccharide chains of gp120, which may explain the broad anti-viral activity of AH against HIV strains. Our findings emphasize that high-mannose type sugar chains of HIV-1 gp120 are important targets for therapy and prophylaxis against HIV/AIDS.

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