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Original article

Thrombin activates envelope glycoproteins of HIV type 1 and enhances fusion

Hong Ling [a,b], Peng Xiao [a,b], Osamu Usami [a], Toshio Hattori [a,*]

[a] *Division of Allergy and Infectious Diseases, Department of Internal Medicine, Graduate School of Medicine, Tohoku University, 1-1Seiryo-Machi, Aoba-ku, Sendai 980-8574, Japan*
[b] *Department of Microbiology, Harbin Medical University, Harbin, 150086, China*

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Abstract

To elucidate the roles of serine proteases, including thrombin, in HIV infection, we treated H9 cells infected with HIV-1 LAI virus (H9/IIIB) with four different proteases (thrombin, cathepsin G, trypsin and chymotrypsin) and observed their effects on functional epitopes on both gp120 and gp41 by using flow cytometry. Monoclonal antibodies (MAbs) against the V3 loop, V2 loop, CD4 binding site, coreceptor binding site and gp41 were used. It was found that trypsin decreased the binding of all MAbs except for one MAb against the V3 loop (IIIB-V3-21). Chymotrypsin and cathepsin G did not show any remarkable effect on the antigen expression. On the other hand, thrombin decreased the reactivities of two out of four anti-V3 MAbs and increased the exposure of functional gp120 epitopes including the coreceptor binding site and CD4 binding site. Thrombin also increased the expression of 2F5 antigen (a neutralizing epitope of gp41) but had no effect on other gp41 epitopes. The effect of trypsin or thrombin on HIV-induced cell fusion was examined through co-culturing H9/IIIB and MAGI cells. Trypsin slightly inhibited fusion. Fusion was significantly enhanced in a dose-dependent manner by thrombin, and a 280% increase at 5 U/ml ($P < 0.001$) was observed. In conclusion, thrombin, one of the major inflammatory molecules in blood, facilitates HIV-induced cell fusion, probably by activating gp120.

Keywords: HIV-1; Envelope; Thrombin; Fusion

1. Introduction

HIV infection is facilitated by the presence of inflammation. Vasodilation and vascular permeability are the earliest signs of inflammation, followed by the activation of coagulation cascades, which generate many inflammatory molecules, including thrombin. It is well established that HIV gp120 contains cleavage sites within the most conserved tip of the V3 loop (GPGRAF sequence) and can be cleaved by some serine proteases, such as thrombin [1–3], tryptase TL2 [4] and others [5–7]. However, only a limited number of proteases, such as urokinase-type plasminogen activator (uPA), have been reported to facilitate viral infectivity by unknown mechanisms [8,9].

Recent findings showed that one of the chemokine receptors, in addition to the primary receptor, CD4, interacts sequentially with HIV gp120 and causes fusion between the viral and cellular membranes [6,10,11]. These studies led to the crystallization of gp120 with soluble CD4 and a human monoclonal antibody (MAb) to the coreceptor binding site (BS), and clarified the topology of functional epitopes of gp120. We have previously reported that V3 loop peptides activate gp120 and induce fusion [12]. In the present study, we examined the effects of several proteases on the changes in these functional epitopes on both gp120 and gp41 and analyzed the mechanisms of enhancement of HIV-induced cell fusion by proteases, and found that thrombin increased the exposure of HIV envelope functional epitopes and enhanced the fusion.

Abbreviations: HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; BS, binding site; MFI, mean fluorescence intensity.

* Corresponding author. Tel.: +81-22-717-8220; fax: +81-22-717-8221.
E-mail address: thattori@intl.med.tohoku.ac.jp (T. Hattori).

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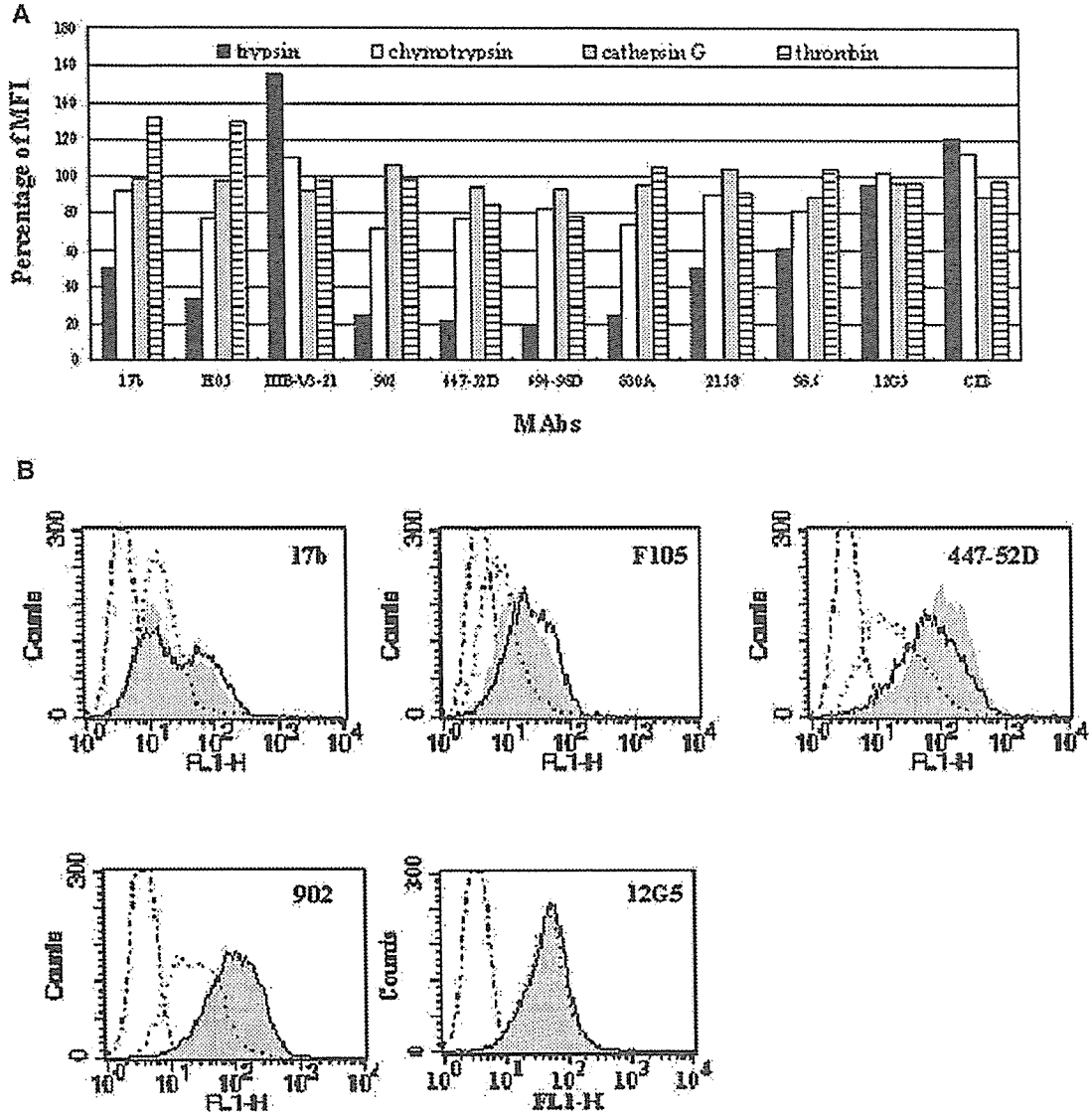

Fig. 1. Effects of the proteases on the binding of MAbs to H9/IIIB cells. (A) The results were expressed as percent of mean fluorescence intensity (MFI) of H9/IIIB cells treated with the proteases compared with the staining when using the corresponding MAb alone. The concentrations of trypsin, chymotrypsin, cathepsin G and thrombin were 5, 10, 5 μg/ml and 10 U/ml, respectively. The MFI of the samples stained with MAbs alone were 33.54, 29.46, 7.03, 116.82, 133.07, 152.02, 62, 24.2, 33.85, 54.52 and 46.6 for 17b, F105, IIIB-V3-21, 902, 447-52D, 694-98D, 830A, 2158, 98.6, 12G5 and OKT3 (CD3), respectively. (B) Representative histograms including the data of 17b, F105, 447-52D, 902, 12G5 as well as the negative control (H9/IIIB cells stained with FITC-secondary MAb alone after treatment with trypsin) are shown. —Thrombin, ····· trypsin, – · – negative control, ▨non-treated.

2. Materials and methods

2.1. Cells and antibodies

The H9/IIIB, H9 cell line, chronically infected with HIV-1 LAI and stably expressing viral envelope but lacking CD4, was maintained in RPMI 1640 (GibcoBRL) supplemented with 10% fetal calf serum (FCS). The HeLa-CD4 long-terminal repeat-beta-galactosidase (MAGI, [13]) cell line was maintained in D-MEM supplemented with 10% FCS, 0.2 mg/ml of G418 and 0.1 mg/ml of hygromycin B (Sigma) as selection reagents. Viabilities of the cells were examined by the trypan blue dye exclusion method and always exceeded 95% when used for experiments.

All the monoclonal antibodies used in the present study are listed in Table 1.

2.2. Serine proteases

Trypsin, chymotrypsin and thrombin were purchased from Sigma (Cat. Nos. are (T1426, C7762 and T9010, respectively.) Cathepsin G was purchased from NCI Biomedicals Inc. (Aurora, Ohio; Cat. No. is 191344).

2.3. The effect of proteases on HIV envelope epitopes

To examine the effect of the proteases on gp120 epitopes on the surface of H9/IIIB cells, 5×10^5 cells were incubated

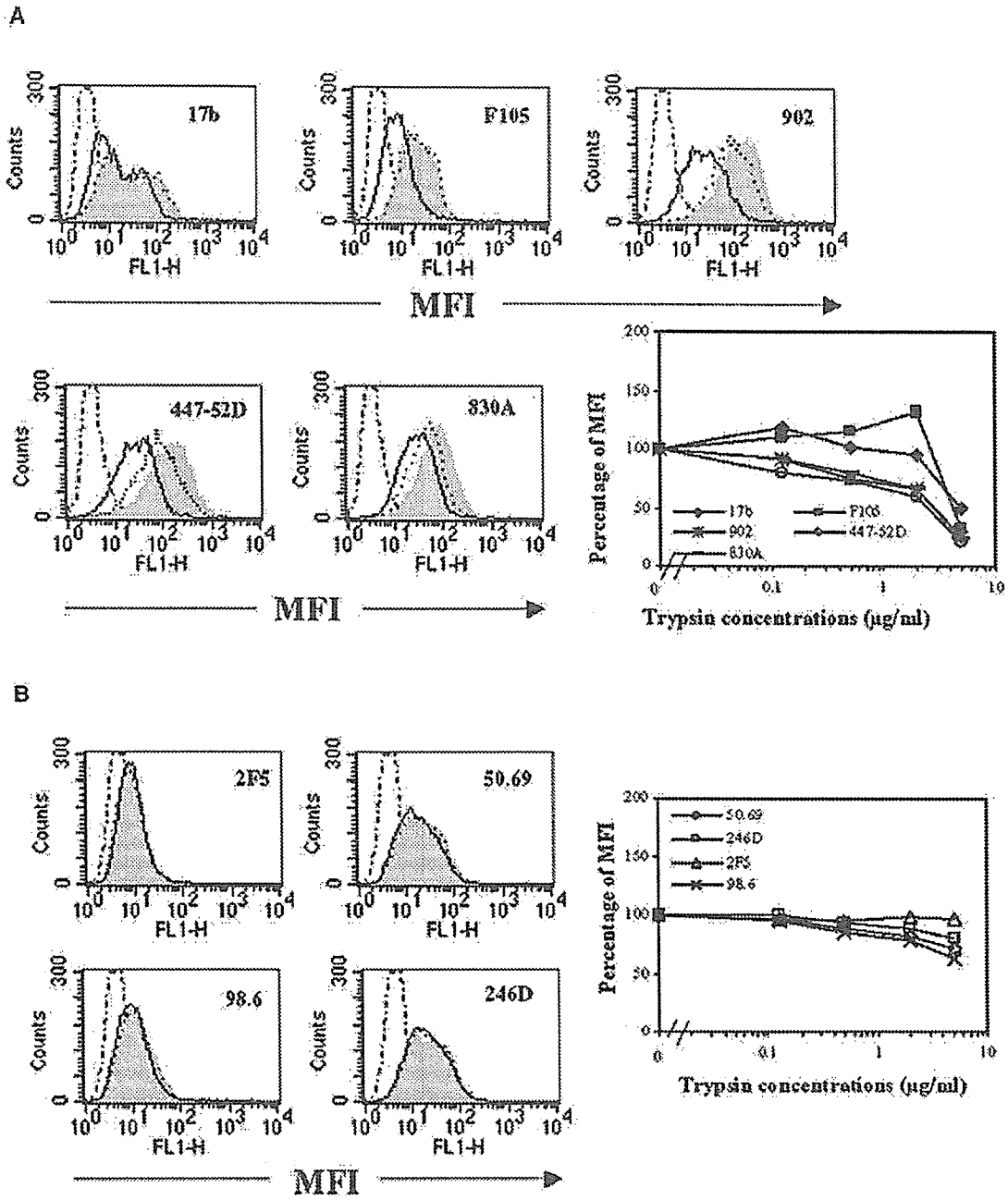

Fig. 2. Effects of trypsin on the binding of MAbs. In A (MAbs to gp120) and B (MAbs to gp41), the effects are expressed as percent of MFI of H9/IIIB cells treated with trypsin compared with MAb staining alone. MFIs of MAbs alone were essentially similar to those in the legend for Fig. 1. —5 μg/ml, ·····0.5 μg/ml, – · – negative control, ▒non-treated.

in the presence of the proteases at various concentrations in 100 μl of PBS (phosphate-buffered saline, 10 mM Na_2HPO4, 2 mM KH_2PO4, 1.37 mM NaCl and 2.7 mM KCl, pH 7.4) at 37 °C for 30 min (for trypsin, chymotrypsin and cathepsin G) or for 2 h (for thrombin). The highest concentrations of the proteases at which viabilities of the treated cells exceeded 95% were chosen. After being washed twice in a washing solution (PBS/0.1% BSA/0.01% NaN_3), the cells were incubated with the MAbs anti-HIV envelope gp120 and gp41 listed in Table 1 at 4 °C for 30 min and washed in the washing solution. Subsequent flow cytometry analysis was performed on FACScan (Becton Dickinson and Co., Mountain View, CA) as described [12]. The controls were samples treated without the proteases and stained with MAbs alone. The results are expressed as percent of control.

2.4. Fusion assay

The fusion assay was performed through co-culturing H9/IIIB and MAGI cells. One day before the experiment,

Table 1
Monoclonal antibodies used in the study

MAb	Core epitope	References
Anti-gp120		
Anti-V3 loop		
447-52D	GPGR	[14]
694-98D	GPGRAF	[14]
902	[a]NT	[15]
IIIB-V3-21	INCTRN	[16]
Anti-coreceptor BS		
17b	Discontinuous	[11,17]
Anti-CD4 BS		
F105	Discontinuous	[18]
Anti-V2 loop		
830A	Conformational	[12]
2158	Conformational	[12]
Anti-gp41		
2F5	ELDKWA	[19]
246D	LLGI	[20]
98.6	Conformational (aa 644-663)	[21]
50-69	Conformational (aa 579-613)	[21]
12G5	Human CXCR4 Discontinuous	[22]
CD3	Human CD3, Discontinuous	

[a] NT: not tested.

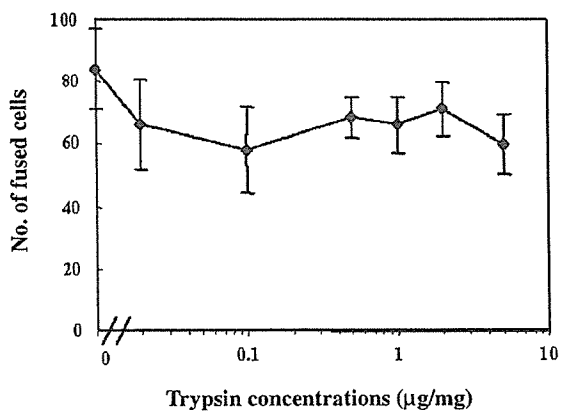

Fig. 3. Effect of trypsin on cell–cell fusion. The numbers of fused cells from six wells (mean ± S.D.) at one concentration of two experiments from three different experiments with similar results are shown.

MAGI cells were plated into the wells of a 48-well plate at 2×10^4/well. On the experiment day, 3×10^4 H9/IIIB cells were added into the wells in 300 μl of RPMI 1640 supplemented with 2% FCS and maintained for 20 h at 37 °C. The fusion is indicated by the formation of multinucleated cells in blue developed by staining with 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal, Sigma) [13,23]. To assess the effect of the proteases, H9/IIIB cells were incubated with thrombin or trypsin of various concentrations as described above. After being washed, the cells were added to MAGI cells in triplicate. After co-culturing for 20 h, the cells were stained with X-gal to detect the syncytia, and all fused cells in a well were counted under an inverted microscope in triplicate. All the experiments were repeated 2–4 times. The data obtained were analyzed by the statistical program SPSS for Windows, version 10.0. Comparisons of the numbers of positive cells with and without protease treatment were evaluated by using ANOVA and HSD Tuckey test.

3. Results

3.1. The serine proteases act in various manners to influence HIV envelope

It was found that the proteases affect envelope epitopes in various manners, as shown in Fig. 1. Trypsin decreased the binding of all including the MAbs against V3 loop (with one exception, MAb IIIB-V3-21), V2 loop, CD4 BS (F105), coreceptor BS (17b) and gp41 epitopes. In contrast, when we examined their effects on cell surface molecules using MAbs anti-CXCR4 (12G5) or -CD3, trypsin did not affect the binding of these MAbs. MAb IIIB-V3-21, which does not bind to gp120 on untreated H9/IIIB, reacted after the cells were treated with trypsin. Chymotrypsin had a much weaker effect on the envelope and no effect on the binding of MAb IIIB-V3-21. Cathepsin G showed no apparent effect on the binding of any MAbs to the HIV envelope nor to the cellular CXCR4 and CD3 epitopes (Fig. 1). However, thrombin decreased the binding of MAbs against the tip of the V3 loop (447-52D and 694-98D), but not that against other V3 regions. On the other hand, it increased the binding of MAbs anti-coreceptor BS (17b) and -CD4 BS (F105). Cellular epitopes were again not affected by thrombin treatment.

3.2. Trypsin did not enhance HIV env-induced cell to cell fusion

As described above, trypsin and thrombin had different effects on the binding of MAbs against CD4 and coreceptor binding sites. We examined the effects using different concentrations of trypsin. The binding of MAbs against V3 (902, 447-52D) and V2 loop (830A) were decreased, while that of MAb F105 was slightly increased in a dose-dependent manner. On the other hand, the binding of MAb 17b was rather decreased (Fig. 2A). The binding of the MAbs against cluster I and II of gp41 decreased while that of MAb 2F5, whose epitope is located at the C-terminal of gp41, did not change (Fig. 2B).

Because the results showed a slight increase of exposure of CD4 BS by trypsin treatment, we next examined its effect on cell to cell fusion. Trypsin did not enhance but rather slightly inhibited fusion (Fig. 3).

3.3. Thrombin increased HIV-induced cell fusion by exposing receptor BS in gp120 and gp41

The effect of thrombin at different concentrations on the MAb binding was also examined. There was no marked

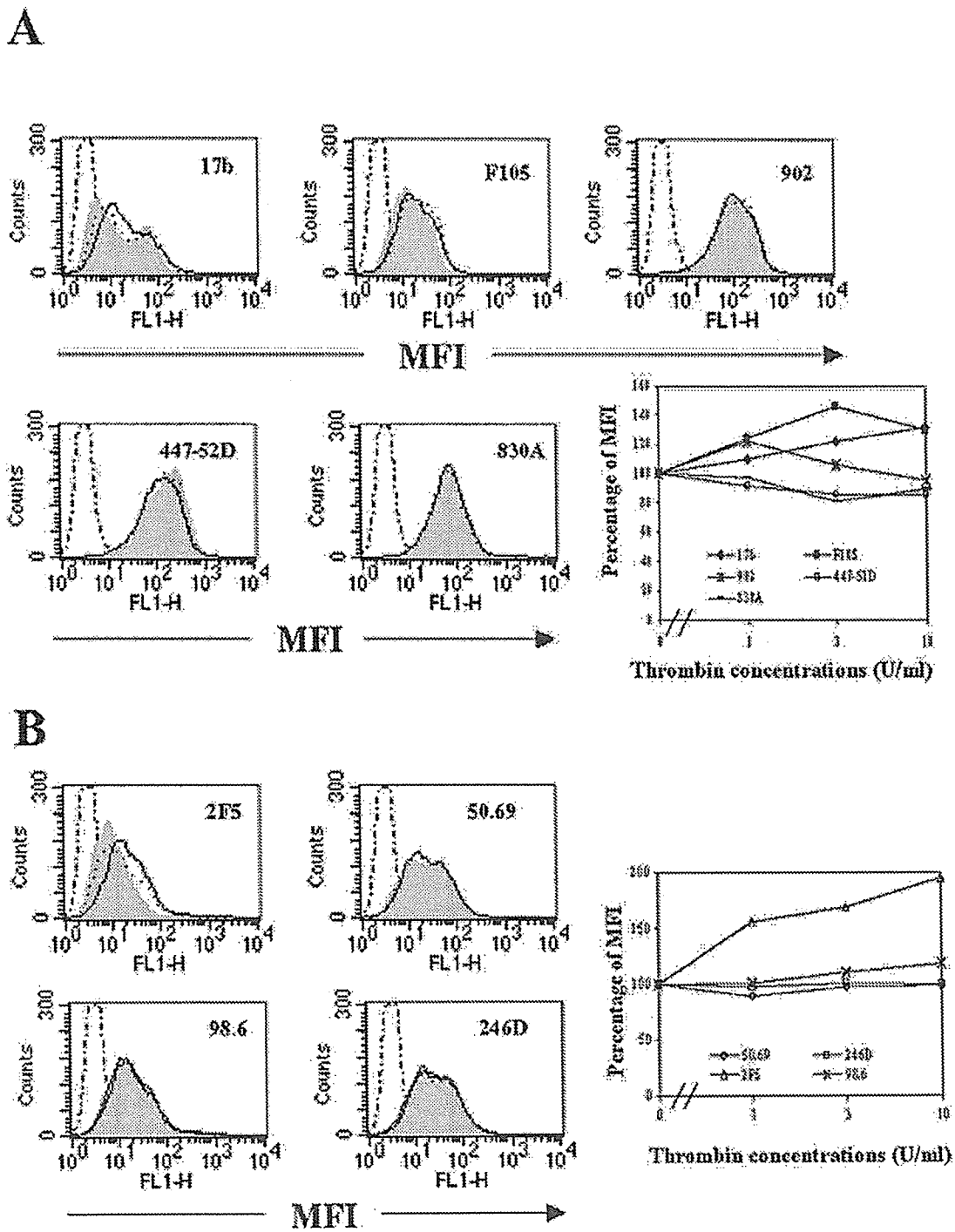

Fig. 4. The effect of the thrombin on the binding of MAbs. In A (MAbs to gp120) and B (MAbs to gp41), the effects are expressed as percent of MFI of H9/IIIB cells treated with thrombin compared with MAb staining alone. —10 U/ml, ····· 3 U/ml, negative control, – · – non-treated.

effect on the V3 loop, and MAb 902 reacted similarly before and after treatment. The reactivity of MAb 447-52D was decreased. (Fig. 4A). It was shown that both CD4 and coreceptor BSs were induced by thrombin. When the binding of the MAbs against gp41 was examined after the treatment with thrombin, that of MAb 2F5 epitope increased significantly but those of the other MAbs did not change (Fig. 4B). The fusion assay was carried out as described above after H9/IIIB cells were treated with thrombin for 2 h. Fusion was significantly enhanced in a dose-dependent manner (Fig. 5). Enhancement of fusion reached a peak and a 280% increase was observed at 5 U/ml, but the extent of enhancement rather

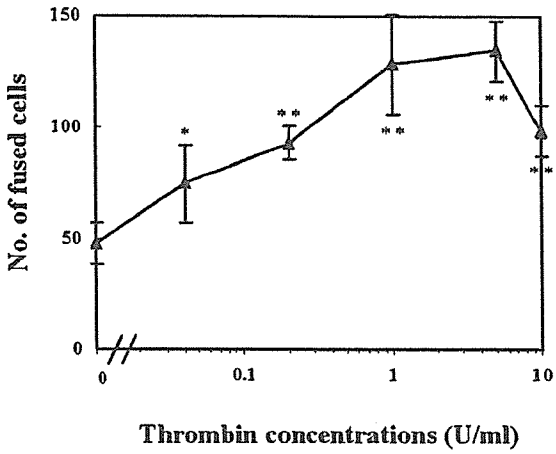

Fig. 5. Effect of thrombin on cell–cell fusion. The numbers of fused cells from six wells (mean ± S.D.) at one concentration of two experiments from three different experiments with similar results are shown. *, $P < 0.05$; **, $P < 0.001$.

decreased when the amount of protease was increased up to 10 U/ml. The enhancement was statistically significant, as shown in Fig. 5.

4. Discussion

In the present study, we evaluated the effects of several serine proteases on functional epitopes expressed by gp120 and gp41 of HIV-1 infected cells. We chose trypsin as a representative of the intestinal tract and thrombin as that of inflammatory foci because they are the predominant proteases of the preferred places for HIV entry into the human body. The results showed that thrombin enhanced HIV-induced cell fusion, probably through exposing receptor binding sites and the conformational change of gp41.

The present study showed that thrombin specifically decreased the binding of MAbs against the tip of the V3 loop but not that to other V3 regions. This phenomenon could be explained by the specific cleavage, because it has been confirmed that thrombin can cleave HIV gp120 at the conserved tip (GPGR ↓ AF) of V3 loop [1,24]. The epitope of MAb 902 has not been clarified and we do not know why its binding did not change after treatment. However, an increase in the exposure of both CD4 and coreceptor BS in gp120 and an enhancement of cell–cell fusion mediated by the HIV IIIB envelope by thrombin treatment was also found. Of note, the treatment increased the binding of MAb 2F5, a well-known neutralizing MAb against gp41, suggesting conformational changes of gp120/41, though the role of this epitope for fusion should be elucidated. It has been suggested that the HIV envelope is a functional conformation in which receptor binding sites are partially or mainly occluded by surface structures [25]; especially, the co-receptor binding site is probably occluded by V2 and/or V3 structures, and its binding to gp120 is associated with virus entry [26]. Therefore, proper cleavage of the surface loop (s) may expose these occluded sites, facilitating HIV interaction with the receptors. Trypsin treatment did not enhance HIV-induced cell fusion, but decreased the binding of MAbs against gp120 and gp41, though it increased the exposure of CD4 BS of gp120. The reasons for different behaviors of thrombin and trypsin in HIV infection are not clear, but could be explained by differences in the cleavage manner, because there are as many as seven possible cleavage sites in the V3 loop for trypsin [27], while there is only one for thrombin. The multiple cleavage sites of trypsin may explain diminished expression of gp120 epitopes. While that of CD4 BS rather increased, suggesting that the CD4 BS might be relatively resistant to trypsin, probably because it contains a well-conserved glycosylation site [28]. Biological activities of thrombin on T cells through interaction with its receptors have recently been reported but it is controversial whether it could stimulate cells through thrombin receptors or not [29,30]. Present results also showed that fusion enhancement is less likely through thrombin receptor signaling on H9 cells because (1) the effect was observed only 120 min after treatment with thrombin; (2) expression of other cell surface molecules including CD3 and 12G5 was not affected.

Recent studies suggested that thrombin is a physiologic mediator of inflammatory events, as it can induce some inflammatory factors such as RANTES at inflammation places as well as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). It therefore may play important roles in the migration of inflammatory cells [31,32]. In addition, it was reported recently that it can also increase the adhesion of macrophages [32]. On the other hand, inflammation at the sites of HIV entry into the body facilitates infection, such as in the case of STDs. Taken together, thrombin may play important roles in HIV infection as well as in the HIV-induced pathogenesis.

This is the first report of an enhancement of HIV-induced cell fusion through the exposure of functional structures of the viral envelope to thrombin. This finding may help us to understand HIV entry and additive effects caused by other microbes in the pathogenesis of HIV infection.

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The N-Terminal of the V3 Loop in HIV Type 1 gp120 Is Responsible for Its Conformation-Dependent Interaction with Cell Surface Molecules

HONG LING,[1,2] OSAMU USAMI,[1] PENG XIAO,[1,2] HONG-XI GU,[2] and TOSHIO HATTORI[1]

ABSTRACT

The V3 loop of HIV-1 gp120 plays an important role in the interaction of the viral envelope with cellular coreceptors and/or with other cell surface molecules. To clarify this interaction we used a panel of monoclonal antibodies (MAbs) against V3 loop and synthetic looped V3 peptides V3-BH10, V3-ADA, and V3-89.6, derived from the V3 regions of the BH10 clone of IIIB (X4-tropic), ADA (R5-tropic), and 89.6 (R5X4-tropic), respectively. A linear mutant peptide, V3-BH10/CA, was also synthesized as a control. Biotinylated V3-BH10, -BH10/CA, and -ADA were also made. The binding abilities of the biotinylated and nonbiotinylated peptides to various types of cells were investigated by using flow cytometry. Subsequently, the principal region of the V3 loop involved in cell surface binding was analyzed by using MAbs against the tip (447–52D and 694–98D), N-termini (IIIB-V3-21) or C-termini (IIIB-V3-01) of the V3 loop in flow cytometry and enzyme-linked immunoabsorbent assay. We demonstrate that looped V3 peptides of both X4 and R5X4 HIV (V3-BH10 and V3-89.6) can bind to various types of cells irrespective of their CD4 and/or coreceptor expression in a conformation-dependent manner. In contrast, the V3 loop of R5 HIV (V3-ADA) can scarcely bind to the cells. Using MAbs whose epitopes cover the entire V3 loop we found that MAb IIIB-V3-21 can react with plate-bound but not cell-bound peptides, and the MAb blocked biotin-V3-BH10 binding suggesting that the N-terminal of the V3 loop interacts directly with cell surface molecule(s).

INTRODUCTION

ATTACHMENT OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) to the cell surface is the initial step of its interaction with target cells. The third variable region (V3 loop) of the viral envelope glycoprotein gp120 has been confirmed to be one of the crucial elements in this interaction with cellular molecules and has therefore been studied extensively.[1–6] Previous studies have demonstrated that the gp120 V3 loop is a major determinant of HIV-1 tropism and coreceptor usage.[7] It has not been clarified how the V3 loop is additionally involved in chemokine receptor usage, but the process is likely complex because both X4 and R5 use a conserved bridging sheet for chemokine receptor binding.[8] Recently, using native proteins, it has been found that the affinity of gp120/CCR5 is higher than that of gp120/CXCR4. However, gp120/CXCR4 interaction analysis was very difficult due to high background.[9] We have demonstrated that biotinylated V3 loop peptides can bind to the cell surface and have characterized the binding proteins on the cell membrane,[3,4] but the epitopes of the envelope responsible for the binding to cells have not been determined yet. Recently, two functionally distinct regions of the V3 loop, designated as the stem and the crown, were shown to be required for gp120 binding to CCR5, but the V3 crown alone determines the coreceptor specificity of the virus.[10] Site-directed mutagenesis carried out on three such V3 residues revealed that the Arg-298 of HIV-1 gp120 has an important role in CCR5 utilization.[11] In these studies soluble gp120 or chimeric viruses were used.

Recently, we have found that V3 loop peptides can enhance the entry of their own HIV strains, probably by the interaction of the V3 loop with gp120 itself, because the peptide exposed the coreceptor binding site of gp120.[12] We also found that pretreatment of the target cells with V3 peptides followed by removal of the peptides also enhanced infection, indicating that

[1]Division of Allergy and Infectious Diseases, Department of Internal Medicine, Graduate School of Medicine, Tohoku University, Aoba-ku, Sendai 980-8574, Japan.
[2]Department of Microbiology, Harbin Medical University, Harbin, 150086, China.

binding of the peptides to target cells also plays a role in this enhancement, though the mechanisms are not clear (unpublished data). In the present study, we attempted to find the binding characters and the crucial region within the V3 loop responsible for its interaction with cell surface molecule(s) by using synthetic V3-looped peptides. We found that the V3 loop binds to the cell surface in a confirmation-dependent manner and its N-terminal is responsible for this interaction.

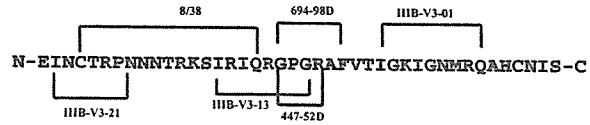

FIG. 2. Epitopes for anti-V3 loop monoclonal antibodies. The amino acid sequence is from the V3 loop of HTLV-1 IIIB gp120.

MATERIALS AND METHODS

Synthetic V3 peptides

The amino acid sequences of the synthetic peptides derived from the third variable region (V3 loop) of X4 (IIIB), R5 (ADA), and R5X4 (89.6) HIV-1 strains are listed in Figure 1. V3-NNT24 was synthesized as described previously.[6] Other peptides were synthesized by Peptide Institute, Inc. (Osaka, Japan) using an automatic peptide synthesizer 430A (PE Applied Biosystems, Foster City, CA) via well-established methods. A disulfide bond was made for V3-BH10, V3-ADA, and V3-89.6 by oxidation by adding a 0.1 M I2 solution of methanol. The oxidation reaction was stopped by adding ascorbic acid and the reaction mixture was immediately used for the next purification procedure using reverse-phase high-performance liquid chromatography (RP-HPLC) under a 16–36% linear gradient of acetonitrile-H_2O (0.1% TFA) for 80 min. The desired oxidized product appeared later than the thiol material. The purified peptides were collected and lyophilized. The homogeneity and disulfide bond formation of the products were confirmed by analytical HPLC, amino acid analysis, and mass spectrometry (MS). All biotinylated V3 peptides including biotin-V3-BH10 (bio-BH10), biotin-BH10/CA (bio-CA), and biotin-V3-ADA (bio-ADA) were synthesized by conjugating a biotin molecule at the N-terminal of the corresponding V3 peptides. The biotinylation was carried out by manual procedures. The N-terminus free peptide resin, biotin, 1-hydroxybenzotriazole, and dicyclohexcylcarbodiimide were mixed for 5 hr at room temperature in 20 ml of *N*-methylpyrrolidone. After no free N-terminus amino group was detected using the ninhydrin reaction, the reaction mixture was washed with methylenechloride and dried. The biotinylated peptide resin was used for the deprotection procedure. The subsequent steps for oxidation, purification, and examination of the peptides were exactly the same as those for nonbiotinylated V3 peptides. The purities of all peptides exceeded 95%. The MS analysis showed a single peak of each peptide and the peptides had their theoretical molecular weights. Biotinylated human parathyroid (PTH)-related protein (1–34), bio-PTH/RP, was purchased from Peninsula Laboratories, Inc. (San Carlos, CA) and used as a negative control for the biotinylated V3 peptides.

```
V3-BH10 (X4)      EINCTRPNNNTRKSIRIQRGPGRAFVTIGKI GNMRQAHCNIS
V3-BH10/CA        ---A---------------------------- -------A---
V3-NNT24                  ------------------------ -
V3-89.6(R5X4) ESVV-----------RRLS-  ------YARRN-IGDI--------
V3-ADA (R5)       ---------------H-  ------Y-T-E-IGDI--------
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FIG. 1. Amino acids sequences of V3 peptides used in the present study. The dashes represent the identities of the residues of V3-BH10.

Monoclonal antibodies

The monoclonal antibodies (MAbs) used in the present study are shown in Figure 2. Mouse MAbs IIIB-V3-21, IIIB-V3-13, and IIIB-V3-01 (all IgG_1) were provided by Dr. J.D. Laman through the National Institute for Biological Standards and Control (NIBSC, UK).[13,14] MAb 8/38 (rat IgG_{2a}) was provided by Dr. C. Shotton and Dr. C. Dean through NIBSC.[15] Human MAbs 447-52D (IgG_3) and 694-98D (IgG_1) were provided by Dr. Zolla-Pazner.[16,17] One conformation-dependent anti-V3 loop mouse MAb, 902 (IgG_1), which is specific to BH10, was provided by Dr. B. Chesebro, through the NIH AIDS Reagent and Reference program.[18] Purified mouse IgG_1 (Nichirei, Tokyo, Japan) and human IgG_1 (Calbiochem, San Diego, CA) myeloma proteins were used as controls.

Cell lines and culture

A cell line CEM.CCR5 (expressing both CXCR4 and CCR5, provided by Dr. Maeda, Kumamoto University) and its parental T cell line, and HOS.CXCR4 (expressing CXCR4, provided from Dr. Landau through the NIH AIDS Reagent Reference Program) and its parental cell line, HOS, were used. CEM, SupT1, Molt-4, human peripheral blood mononuclear cells (PBMC), and monocytes-derived macrophages (MDM) were also used. Human PBMC and MDM were obtained as previously described[19] with slight modifications. Briefly, PBMC were isolated from healthy HIV-1-seronegative donors using Ficoll-Paque Plus (Amersham Pharmacia BiotechAB, Uppsala, Sweden). The PBMC were used immediately for fluorescence-activated cell sorting (FACS) analysis by staining with the biotinylated V3 peptides. To obtain MDM, enriched monocytes from PBMC were incubated at 37°C for 24 hr in a plastic dish coated with human AB serum. One day later, nonadherent cells were removed, and the adherent cells were cultured for 5 days in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 5% giant cell tumor-conditioned medium (Igen, Inc., Rockville, MD). Adherent macrophages were treated with 0.5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 10 min to harvest the cells and stained with the biotinylated peptides.

Biotinylated V3 peptide binding

One half million of the cells were incubated with bio-BH10, bio-CA, bio-ADA, or bio-PTH/RP diluted in RPMI 1640 containing 0.1% bovine serum albumin (BSA), 0.01% sodium azide, and 25 mM HEPES (incubation solution) at the various

concentrations indicated in the figure legend for 40 min at 4°C. The cells were washed twice with PBS containing 0.1% BSA, 0.01% sodium azide (washing solution) and stained with R-phycoerythrin conjugate streptavidin (RPE-streptavidin, Molecular Probes, Inc., Eugene, OR) in the same buffer for 30 min at 4°C. After removing the RPE-streptavidin by washing twice, the cells were resuspended in 2% formaldehyde (fixing solution) and analyzed by using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). The baseline bindings consisted of those from the cells treated with the highest concentration of the corresponding nonbiotinylated V3 peptides.

Binding of nonlabeled V3 peptides to cell surface detected by MAbs anti-V3 loop

The cells were pretreated with V3 peptides including V3-BH10, their linear mutants, V3-BH10/CA and -NNT24, as well as V3-ADA, 89.6 for 1 hr at 37°C followed by washing. Cell-surface-bound peptides were developed through staining with antibodies against the V3 loop (Fig. 2). Fluorescein isothiocyanate (FITC)-conjugated second antibodies were used to detect the anti-V3 MAbs. Flow cytometry was carried out as described above. Using this indirect binding assay, we calculated the percentage increase of MFI as follows: % increase = (MFI of peptide treated cells − MFI of control cells)/MFI of control cell × 100.

Reaction of anti-V3 MAbs with V3 loop peptides in ELISA

V3 peptides dissolved in PBS at 1 μM were coated on a 96-well enzyme-linked immunosorbent assay (ELISA) plate (NALGE-NUNC International Corp, CA) as described.[20] After being washed in PBS-T (PBS containing 0.05% Tween 20), the plates were blocked by incubating with 0.5% BSA for 1 hr at 37°C. Then 5 μg/ml of each MAb against the V3 loop in 100 μl was added to one of three wells and incubated for 2 hr at 37°C. Bound MAbs were detected by using horseradish peroxidase-linked anti-human, anti-mice, or anti-rat antibodies followed by incubation with 1,2-phenylenediamine dihydrochloride (OPD) (DAKO) working solution and the optical densities (OD) at a wavelength of 450 nm were detected.

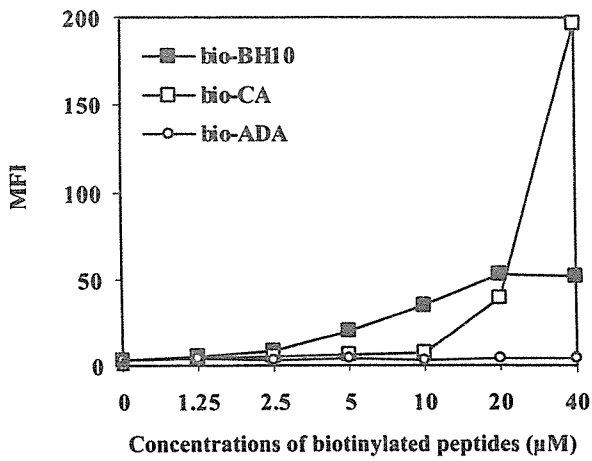

FIG. 3. Direct binding of biotinylated V3-BH10, V3-BH10/CA, and V3-ADA to CEM.CCR5 cells. The cells are expressed as mean fluorescence intensity (MFI) from one representative experiment of three independent ones.

TABLE 1. BINDING OF BIOTINYLATED V3 PEPTIDES TO CELL SURFACE

	bio-BH10	*bio-ADA*	*bio-PTH/RP*
T cell lines			
CEM	36.98	1.15	0.17
CEM.CCR5	72.72	0.12	0.03
SupT1	7.67	0.21	0.28
Molt4	322.4	0.33	0.96
HOS	76.17	1.55	0.54
HOS.CXCR4	94.62	1.21	0.62
PBMC			
Lymphocyte	7.41	0.18	0.04
Monocyte	81.84	2.89	0.2
MDM	23.67	2.65	0.24

Effects of MAbs anti-V3 loop on bio-BH10 binding

Bio-BH10 (7.5 μM) and one of the MAbs anti-V3 loop at different concentrations were incubated at 4°C for 15 min. Then the mixture was added to CEM.CCR5 cells. After being incubated at 4°C for 30 min, the cells were washed and developed by RPE-streptavidin as described above. Mouse and human IgG_1 myeloma proteins were used as the negative controls.

RESULTS

Various characteristics of direct binding to the cell surface among the V3 loop peptides derived from various HIV strains

We used biotinylated V3 peptides to determine direct binding of the V3 loop as the peptides can mimic, at least partially, the functions of the natural V3 region of gp120. Direct bindings of biotinylated V3-BH10 and V3-BH10/CA to CEM.CCR5 cells are shown in Figure 3. The binding of bio-BH10 was dose dependent at concentrations ranging from 1.25 to 40 μM (Fig. 3). However, mean fluorescence intensity (MFI) of bio-CA at 40 μM was markedly increased (400%) over that at 20 μM. Similar results were also seen in other independent experiments, indicating that the binding of bio-CA could be less specific at this high concentration. It should be noted that nonbiotinylated peptides cannot block the binding of biotinylated peptides as described previously.[4] It was also found that bio-BH10 can bind to not only T cell lines, but also to lymphocytes and monocytes (gated from PBMC) as well as MDM. In contrast, bio-ADA binding to monocytes from PBMC was very weak and the bindings to lymphocytes and other cell lines were not significant (Table 1).

To investigate the binding characteristics of V3 peptides extensively, we developed a new method, i.e., an indirect binding assay. Using this assay, the bindings of nonlabeled V3-89.6 as well as V3-BH10 were detected by using MAbs anti-V3 loop tip (447-52D and 694-98D), and MFI given by both MAbs to

bound V3-BH10 and V3-89.6 were similar (Fig. 4). In addition, V3-89.6 as well as V3-BH10 bound to all the cell lines examined (CEM, HOS, and HOS.CXCR4) (data not shown).

The results that showed more than 30% of MFIs increase compared to no peptides were considered positive. Based on these criteria, weak binding of V3-ADA was implicated.

Binding of V3 loop peptides is conformation dependent

When we compared the binding of looped V3 BH10 and its linear mutant, V3-BH10/CA to the cell surface, we found that the binding degree of the looped form is stronger than that of the linear form (Figs. 3 and 4). The linear peptide binds to cells always less than the looped peptide depending on the concentrations and experiments. As shown in one representative result (Fig. 4), the MFIs of V3-BH10 and V3-BH10/CA at 10 μM detected with MAb 447-52D were 9.5 and 6.9, respectively, and those at the same concentration detected by MAb 694-98D were 15 and 9, respectively. In addition, the binding of V3-NNT24, the linear and shorter form of V3-BH10, was scarce (Fig. 4). We also compared the net charges and found that they were similar: net charges of V3-BH10, V3-BH10/CA, and V3-NNT24 were 6.6, 6.8, and 5.75, respectively (pH 7.5).

N-terminal of V3 loop is responsible for the binding

To delineate the regions of the V3 loop that are responsible for its binding to cells, we used the MAbs anti-V3 loop overlapping the entire region (Fig. 2). A method for detecting cell-bound peptides was developed as described in Materials and Methods. It was found that all MAbs except MAb IIIB-V3-21 can detect cell-bound V3-BH10 (Fig. 5). MFIs given by MAbs corresponding to different regions within the V3 loop varied. The highest binding was shown with MAb 447-52D, which reacts with the tip of the V3 loop, while the binding extent of the C-terminal of the V3 loop was weaker as shown from the result with MAb IIIB-V3-01. When MAb IIIB-V3-21, whose epitope is exactly the N-terminal, was used to examine the binding, neither V3 peptides including V3-BH10 as well as V3-89.6 could be detected. CEM.CCR5 was used in the experiments but the results were essentially similar when CEM and HOS cell lines were used.

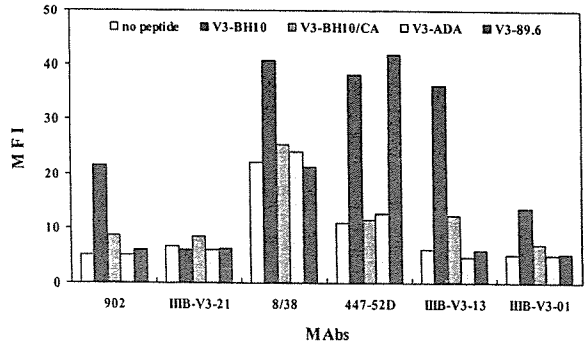

FIG. 5. Detection of cell-bound V3 peptides by using MAbs anti-N, anti-tip, and anti-C regions of the V3 loop. CEM.CCR5 cells were incubated with the peptides followed by development using anti-V3 MAbs as described in Materials and Methods. The result is one representative experiment of three independent ones.

In contrast, when the MAbs were used to examine the plate-bound V3 peptides, not only the MAbs anti-tip or C-terminal of the V3 loop but also IIIB-V3-21 could react specifically with the V3 peptides in accordance with epitope specificity (Fig. 6).

Blocking of bio-BH10 binding to CEM.CCR5 cells by MAb IIIB-V3-21

The effects of the MAbs anti-V3 loops on bio-BH10 bindings to cells were examined at different concentrations ranging from 3 to 100 μg/ml. None of the MAbs anti-V3 loop inhibited the bindings of bio-BH10 except MAb IIIB-V3-21. The inhibitory effect was apparently dose dependent (Fig. 7).

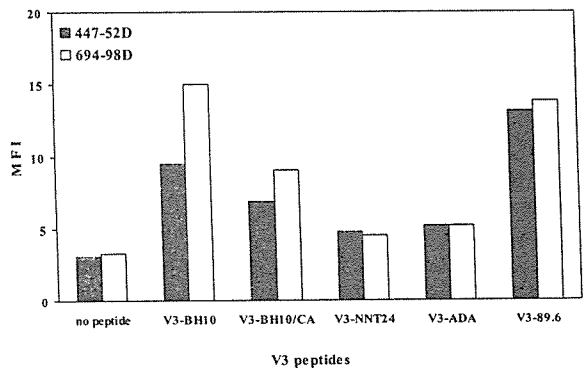

FIG. 4. Binding of nonlabeled V3 peptides to CEM.CCR5 cells detected by using MAbs against the tip of the V3 loop as described in Materials and Methods. The results are expressed as mean fluorescence intensity (MFI) from one representative experiment of three independent ones. The calculated percentage increase comparing to that of no peptides is expressed at the top of each bar.

DISCUSSION

In the present study, we have demonstrated that the looped V3 peptides of both X4 and R5X4 HIV (V3-BH10 and V3-89.6) can bind to various types of cells irrespective of their CD4 and/or coreceptor expression. In contrast, the V3 loop of R5 HIV (V3-ADA) had low binding ability. The variety of binding target cells of the X4 HIV V3 loop may indicate a strong attachment ability, which would facilitate HIV spreading broadly *in vivo* as well as the rapid progress of AIDS. One reason for the stronger binding of the X4 and R5X4 V3 loops could be that their positive charges were higher than that of R5. Probably they could bind to negatively charged molecules on the cell surface. However, they can bind specifically to cellular molecules, too, such as coreceptors, because the V3 loop specifically inhibits the binding of MAb against CXCR4[21] and some other unknown molecule(s) that we have isolated from the cell membrane.[3,4] Our data based on the looped and linear V3 peptides, whose net charges are almost identical, showed that they have different binding ability because at concentrations lower than 20 μM the binding of the looped form was stronger while

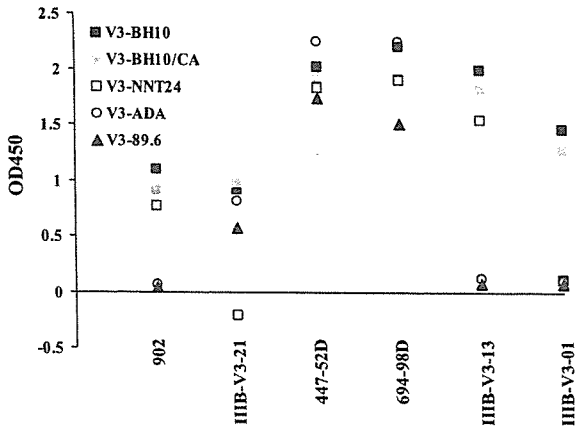

FIG. 6. Detection of plate-bound V3 peptides by using the MAbs anti-V3 loop. The V3 peptides were coated to the microplate and examined by using the MAbs anti-V3 in ELISA. The result is one representative experiment of three independent ones.

at concentrations above 20 μM the linear form had stronger binding. This suggests that the binding of looped peptides observed at lower concentrations could be specific and conformation dependent, and less affected by their positive charges, but that of linear peptides observed at high concentration could be less specific and dependent on the charges.

Recently, it was found that within V3 loop, the tip and stem function differently in terms of the interaction with CCR5 and the determination of cell tropism.[10] The V3 stem alone mediates soluble gp120 binding to the CCR5 N-terminal. In contrast, both the V3 stem and tip are required for soluble gp120 binding to cell surface CCR5. Within the context of a virion, however, the V3 crown alone determines coreceptor usage. The above contradictory phenomena indicate the presence of an unknown cooperative interaction of the V3 crown and stem with distinct regions of CCR5 to mediate viral entry. From a very recent report we know that the V3 crown and C4 residues are relatively dispensable for cell–cell fusion, although some residues may be involved in the regulation of early postentry steps in viral replication.[22] In contrast, seven highly conserved residues located in the V3 stem were reported to be critical for CCR5 utilization in subtype C of HIV-1. In an earlier study it was also found that a highly conserved arginine residue of the V3 N-terminal plays an important role in subtype CCR5 utilization and that this highly conserved arginine may contribute to the functional convergence of chemokine receptor utilization.[11,23] In the present study, we also found that the V3 loop lost reactivity to MAb to the N-terminal epitope only when it bound to cells but not on ELISA plates. This phenomenon could be explained in two ways: (1) the N-terminal is responsible for direct cell-binding or (2) the epitope could be occulted by the conformational change after the V3 loop binds to cells. The specific inhibition of bio-BH10 binding by MAb IIIB-V3-21 (Fig. 7) strongly supports the former explanation and these data using synthetic peptides coincided with the results of other investigators who used chimeric viruses for the analysis as described above. In addition, the binding of MAb anti-C-terminal to cell-bound V3 peptide was weaker than that of MAb antitip. This phenomenon also suggests that the C-terminal is partially responsible for its binding as other investigators reported. From the findings of the present study, the N-terminal of the V3 loop might be responsible for the V3 loop binding to the cell surface.

Finally, our peptide binding assay did not demonstrate a strong association between the V3 loop and coreceptors implying the interactions of gp120 and coreceptors could be more complex. Other investigators have shown that the direct physical interactions between CD4-independent HIV-1 gp120 (IIIBx) and CXCR4 are dependent on both the 17b epitope and V3 loop by a biosensor.[24,25] It was clearly indicated that 17b MAb inhibits direct interactions but it is not known whether anti-V3 MAbs inhibited the interaction or not. In addition, delineation of the binding site of CXCR4 to HIV-2 gp120 was clarified using chimeric proteins.[9] These works should and would clarify the epitope in the V3 loop that is responsible for interactions with chemokine receptors.

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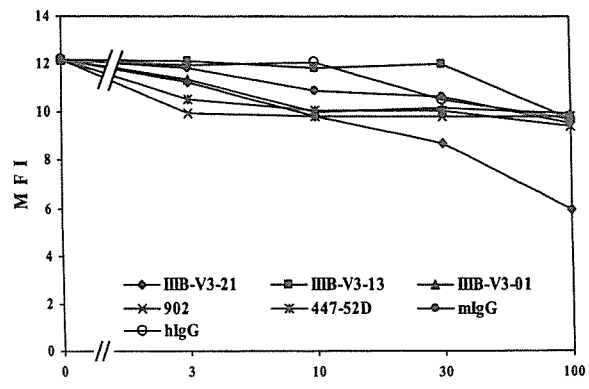

FIG. 7. Blocking of bio-BH10 binding to CEM.CCR5 cells by MAb IIIB-V3-21. The bio-BH10 were mixed with various MAbs anti-V3 loops and the mixture was added to CEM.CCR5 cells. The bound bio-BH10 was detected by flow cytometry as described in Materials and Methods. The result is one representative experiment of three independent ones.

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Address reprint requests to:
Toshio Hattori, M.D.
Department of Internal Medicine
Graduate School of Medicine
Tohoku University
I-1 Seiryo-Machi
Aoba-ku, Sendai 980-8574, Japan

E-mail: thattori@intl.med.tohoku.ac.jp

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Original article

Specific reactions between purified HIV-1 particles and CD4$^+$cell membrane fragments in a cell-free system of virus fusion or entry

Takayuki Harada [a], Masashi Tatsumi [b], Hidehiro Takahashi [a], Tetsutaro Sata [a], Takeshi Kurata [a], Asato Kojima [a,*]

[a] *Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Veterinary Science, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*

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Abstract

The initial step of human immunodeficiency virus type 1 (HIV-1) infection has been studied by Env-mediated fusion or entry assays with appropriate cells expressing CD4 or CXCR4/CCR5 receptors in cultures, where many factors underlying cellular activities likely regulate the fusion/entry efficiency. Here we attempted to develop a more simplified in vitro cell-free fusion/entry reaction that mimics HIV-1 infection in cultures. Membrane fragments of target cells and intact infectious HIV-1 particles were purified, mixed and incubated. The core p24 protein was released from the purified virions and detected by ELISA without detergents in the supernatant of the reaction mixtures. This release reaction proceeded temperature-dependently and in a dose-dependent manner between the virion and membrane fractions, and was specific for HIV-1 Env and CD4. Env-deleted or VSV-G-pseudotyped HIV-1 released little p24, if any. Pretreatment of the membrane fragments with anti-CD4 antibodies inhibited the p24 induction from both X4-tropic and R5-tropic HIV-1. Furthermore, X4 but not R5 HIV-1 reacted with the membrane prepared from intrinsically CXCR4-positive HeLa-CD4 cells, whereas both viruses reacted with that prepared from CCR5-transduced HeLa-CD4 cells, indicating that this cell-free reaction mimics coreceptor usage of HIV-1 infection. Therefore, a potent entry inhibitor of X4 HIV-1, SDF-1α, blocked the release from X4 but not R5 HIV-1. Inversely, a weak entry inhibitor of R5 HIV-1, MIP-1β, partially affected only the release from R5 HIV-1. These results suggest that this cell-free reaction system provides a useful tool to study biochemical fusion/entry mechanisms of HIV-1 and its inhibitors.

Keywords: HIV-1 entry; Membrane fusion; Cell-free reaction; p24 Release

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) infects susceptible cells by fusion of the viral and cellular membranes. The viral envelope (Env) glycoprotein, gp120, interacts with CD4 receptor and a chemokine receptor, CXCR4 or CCR5, on the target cell membrane. Following this interaction, the fusion peptide in Env-gp41 is exposed and inserted in the target lipid bilayer. It brings the viral and cellular membranes together, and membrane fusion takes place at the cell surface. By the fusion process, the viral core composed of the p24 capsid protein is released into the host cell cytoplasm [1,2].

This HIV-1 entry model comes from many studies using Env-mediated fusion assays in cell cultures. Transduction of reporter genes such as *lacZ* has been studied as an index of specific fusion between Env-expressing cells and appropriate CD4$^+$ target cells [3]. Alternatively, amounts of the p24 protein [4] or HIV-1 genome [5] in detergent-treated cell lysates are examined as an indication of HIV-1 core internalization caused by specific interaction of viral Env with the CD4 receptor and CXCR4 or CCR5 coreceptor of cells. Furthermore, fluorescence dequenching [6–8], photosensitized labeling [9] and β-lactamase-based green-to-blue emission shifting [10,11] methods have been described as more sensitive and quantitative assays for HIV-1 fusion/entry to target cells in cultures. Thus, these fusion/entry assays have contributed significantly to the progress in such fields as the discovery of the requisite receptors, the role of receptor and coreceptor densities, and the induction of fusion activities during the Env-receptor interaction [1,2].

Despite the consensus HIV-1 entry model, however, the detailed molecular and biochemical mechanisms behind the

* Corresponding author. Tel./fax: +81-3-5285-1189.
E-mail address: akojima@nih.go.jp (A. Kojima).

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fusion/entry processes are not fully understood. The viral entry process into cells is suggested to involve many complex factors. Hematopoietic stem cells [12] or promonocytic U937 clones [13] were resistant to HIV-1 infection at the steps of virus–cell fusion and entry, although the relevant receptor and coreceptor were expressed in substantial levels. Target cells may intrinsically secrete varying amounts of soluble factors such as CC and CXC chemokines and have different influences on virus entry or infectivity [14–16]. Binding of HIV-1 virions or Env-gp120 to the receptors may activate cellular signaling pathways and result in activation of transcription of the cytokine and chemokine genes [17,18]. Moreover, recent reports suggest that HIV-1 enters cells via a non-specific endocytosis pathway independently of the Env-receptor-mediated fusion step [19–23]. These factors underlying cellular activities make it difficult to analyze the biochemical mechanisms of the sub-steps of HIV-1 entry, such as virion binding, fusion, uncoating and disassembly, due to a limitation of culture systems using whole live cells. Therefore, more simplified in vitro cell-free systems that mimic HIV-1 infection may be favorable for examining the entry mechanisms.

In this study, we attempted to establish an HIV-1 fusion/entry reaction in a cell-free system, where intact infectious virions were separated from non-infectious viruses, mixed and incubated with purified cell membrane fragments. Our results demonstrate that the viral core p24 protein was detected in the supernatant of the reaction mixtures by ELISA without detergents to lyse virions. This release reaction mimicked HIV-1 infection in terms of Env-gp120 dependency, CD4 receptor specificity and CXCR4/CCR5 coreceptor utilization. Therefore, the cell-free fusion/entry reaction system was able to detect the effects of HIV-1 entry blockers.

2. Materials and methods

2.1. Reagents

Mouse anti-human CD4 monoclonal antibody (Leu 3a; IgG_1), mouse monoclonal IgG_1, and recombinant human SDF-1α and MIP-1β were purchased from Becton Dickinson, Chemicon International Inc. and Pepro Tech EC LTD, respectively. Another mouse anti-human CD4 monoclonal antibody (7-4; IgG_1) was prepared from the culture fluid of hybridoma 7-4 [24], by affinity chromatography using Hi-Trap™ Protein G (Amersham Pharmacia Biotech AB).

2.2. Cell lines

Stable MAGIC5 cells were established from HeLa-CD4-LTR-β-Gal (MAGI) cells by transfection with expression vectors for CD4 and CCR5 [25]. MAGIC5 cells and 293T cells [26] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). M8166 cells [27] were maintained in RPMI-1640 medium supplemented with 10% FBS.

2.3. Membrane preparation

Cells grown to semi-confluency were collected with a scraper and centrifuged at 300 × *g* for 5 min. The cell pellet was washed three times with PBS and suspended in a cold hypotonic buffer (10 mM Tris (pH 7.4), 1 mM $MgCl_2$, and 2 μg/ml aprotinin) for 30 min. The suspended cells were homogenized with a Potter–Elvehjem homogenizer (20 strokes), and the degree of cell rupture was checked by microscopy. The homogenate was mixed with 1/10 vol. of 2.5 M sucrose, applied onto 15% Percoll in PBS and centrifuged at 15 000 × *g* for 5 min. The upper layer was collected, washed five times with the sucrose–Tris buffer (0.25 M sucrose, 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, and 2 μg/ml aprotinin) by centrifugation at 4000 × *g* for 15 min and used as membrane fraction. These homogenate and membrane fractions were stored at –80 °C until use.

2.4. LDH assay and immunoblot analysis

Homogenate and membrane fractions were lysed with 1% Nonidet P-40, and the protein contents were determined with a DC protein assay kit (Bio Rad Lab). Activity of cytosolic lactic dehydrogenase (LDH) in the lysates was determined with an LDH assay kit (Azwell Inc.). For immunoblot analysis, each fraction was lysed with an SDS-PAGE sample buffer. Proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore Corp). Gβ, a membrane anchor protein, was detected with anti-Gβ antibody (T-20, Santa Cruz Biotech Inc) at a dilution of 1:300, followed by peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech AB), and an enhanced chemiluminescence detection system (Super Signal West Dura Extended Duration Substrate, Pierce). Band intensities were quantified with an LAS-1000 image analyzer (Image Gauge ver 3.11, Fuji Film Co).

2.5. Virus preparation

NL432 [28] and JRFL [29] viruses were prepared by transfection of 293T cells with the infectious proviral DNA clones by a calcium phosphate coprecipitation method. VSV/NL432env(-) pseudovirus was obtained by co-transfection of a VSV-G expression vector [30] and NL432env(-) proviral DNA [31]. When viruses were propagated, M8166 or MAGIC5 cells were infected for 2 h with viruses (1 ng of p24 per 10^4 cells) in the medium containing polybrene (2 μg/ml) or DEAE-dextran (20 μg/ml), respectively. The infected cells were washed with and cultured in polybrene- or DEAE-dextran-free growth medium. The culture supernatants of infected M8166 and MAGIC5 cells were collected 3 and 4 days later, respectively, clarified by centrifugation at 1500 × *g* for 10 min, and concentrated and purified by ultrafiltration with a large-pore-size membrane (Centricon-100, Amicon Inc.). The virion preparations were stored at –80 °C.

2.6. In vitro fusion assay

Mixtures of purified virus and membrane fractions were incubated at 37 °C for 12 h, unless otherwise mentioned, in a total volume of 20 μl containing 0.5% BSA in PBS. The fusion reaction was stopped with 180 μl of an ice-cold 0.5% BSA–PBS. The diluted mixtures were centrifuged at 100 000 × *g* for 30 min at 4 °C to remove intact virus particles in the precipitate (P_{100}) fraction, and the p24 amounts in the supernatant (S_{100}) fraction were determined with a p24 antigen ELISA kit (Cellular Products Inc.).

2.7. Detection of virus infectivity

The amounts of virions were normalized by p24 ELISA. Virus infectivity was determined by the MAGI assay, as described previously [3]. Briefly, MAGIC5 cells infected with HIV-1 were fixed 2 days later with 1% formaldehyde–0.2% glutaraldehyde for 5 min and stained for 60 min with the staining solution [4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.4 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)]. Blue foci in 96-well cultures were counted under a microscope.

3. Results

3.1. Purification and concentration of intact infectious virions by ultrafiltration

Our initial experiments, in which culture supernatants of 293T cells transfected with HIV-1 DNA clones were mixed with membrane homogenates of target cells, failed to detect significant p24 increases in the cell-free reaction mixtures. Therefore, attempts were first made to concentrate and purify intact infectious HIV-1 particles with less contamination of non-infectious p24, which may hinder the additional p24 release from virions. To obtain a high virus dose, HIV-1 from transfected 293T cells was propagated with MAGIC5 or M8166 cells, and the p24 amounts in the culture supernatant were determined by ELISA. The p24 production by 293T, MAGIC5 and M8166 cells was 93, 193 and 2464 ng/ml, respectively, indicating that M8166 cells are a favorable producer of HIV-1 virions.

The culture supernatant of infected M8166 cells was concentrated to a 1/10 vol. by ultrafiltration with different-pore-size membranes (Centricon-50, -100 and -500), and the filtrate and retentate fractions were tested for p24 amounts (Fig. 1A). There was no detectable p24 in the filtrate fraction of Centricon-50, and all of the p24 antigen was recovered in the retentate fraction. In contrast, only 17% and 11% of p24 was recovered in the retentate fraction of Centricon-100 and -500, respectively, and over 50% of the p24 was removed into the filtrate fraction, indicating that a large amount of p24 was filtered away. To examine whether the retentate fraction contained infectious virions, virus infectivity was determined by

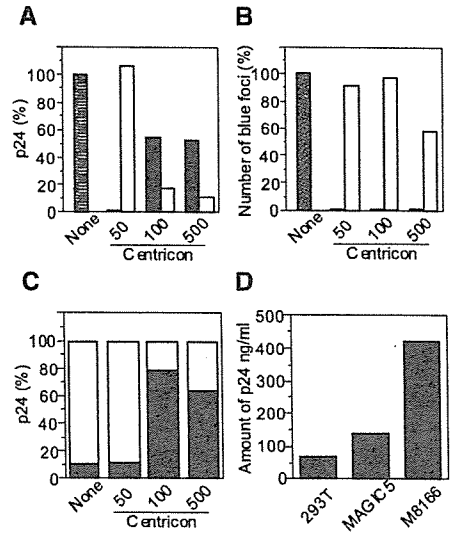

Fig. 1. Purification and concentration of HIV-1 virions by ultrafiltration. The culture supernatant of M8166 cells infected with NL432 virus was ultrafiltrated using Centricon-50, -100 or -500. (A and B) The non-treated culture fluid (shaded bars), the filtrate (closed bars) and retentate (open bars) fractions were examined (A) for p24 amounts by ELISA and (B) for virus infectivity by blue focus assay. (C) The culture fluids and membrane-retentate fractions were ultracentrifuged at 100 000 × *g* for 30 min at 4 °C, and p24 amounts in the P_{100} (closed bars) and S_{100} (open bars) fractions were determined by ELISA. (D) NL432 virus was purified and concentrated from culture fluids of transfected 293T, infected MAGIC5 or M8166 cells by ultrafiltration with Centricon-100. Yields of purified virions were determined by p24-ELISA. In (A), (B) and (C), results are expressed as percentages of the culture supernatant.

blue focus assay. As shown in Fig. 1B, no virus infectivity was detectable in the filtrate fraction. Almost 100% and about 60% of infectious viruses were recovered only in the retentate fraction of Centricon-100 and -500, respectively.

HIV-1 particles in the retentate fraction were precipitated by ultracentrifugation, and p24 amounts in the S_{100} and P_{100} fractions were examined (Fig. 1C). The level of precipitated p24 in the Centricon-50 retentate (11%) was as low as that in the non-purified culture supernatants (12%). In contrast, large proportions (about 80% and 65%) of p24 were precipitated from the Centricon-100 and -500 retentates, respectively. The yields of the intact virions were compared after Centricon-100 purification for 293T, MAGIC5 and M8166 cell cultures (Fig. 1D). Loss of p24 for M8166 cells was about 85%, but the retentate still contained more than 420 ng/ml of p24 that was 6.3- and 2.1-fold greater than that of 293T and MAGIC5 cells, respectively. These results suggest that non-infectious p24 is efficiently removed, and that intact infectious HIV-1 virions are purified and concentrated.

3.2. Purity of membrane preparation

As it was noted that the purity of the membrane preparations affected mainly the target property of HIV-1 attachment in cell-free reactions (data not shown), the homogenate and purified membrane fractions from MAGIC5 cells were ex-

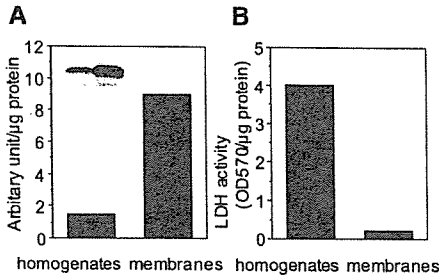

Fig. 2. Identification of cell membrane. Lysates of homogenate and membrane fractions from MAGIC5 cells were analyzed (A) for a membrane anchor protein, Gβ, by immunoblotting with anti-Gβ antibody, and (B) for a cytoplasmic enzyme, LDH, activity in an equal amount of protein. In (A), band intensities shown in the inset were quantified with an LAS-1000 image analyzer.

amined for the contents of a membrane-anchored protein, Gβ and a cytosolic enzyme, LDH, in equal protein amounts.

The result of immunoblotting with anti-Gβ (the inset of Fig. 2A) demonstrates that levels of Gβ were low and high in the homogenate and membrane fractions, respectively. Gβ was quantified by image-scanning and calculated to be concentrated sixfold into the membrane fraction (Fig. 2A). In contrast, specific LDH activity was high and very low in the homogenate and membrane fractions, respectively (Fig. 2B). The membrane fragment preparation contained only 1/20 of the LDH activity in the homogenate. These results suggest that the purity of the membrane fraction may be of a level substantial to function as a fusion partner with purified HIV-1 virions.

3.3. Characterization of cell-free fusion reaction

Purified NL432 virion and MAGIC5 cell membrane fractions were mixed and incubated at 37 °C, and the kinetics of p24 increase in the S_{100} fraction was examined as an indication of the core release from burst HIV-1 virions (Fig. 3A). No significant p24 was detected in S_{100} at 2 h. However, specific p24 release significantly increased at 4 h and was

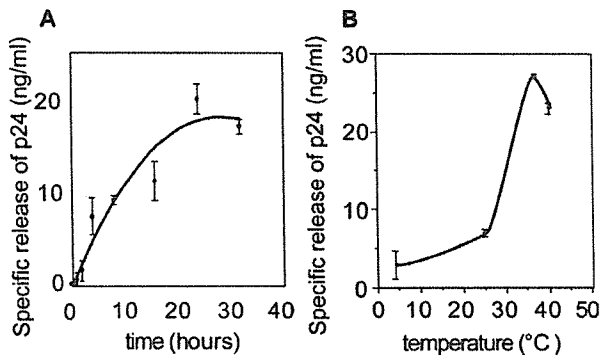

Fig. 3. Kinetics and temperature dependence of p24 release in cell-free reaction system. Membrane fraction (20 μg of protein) from MAGIC5 cells and purified NL432 virions (4 ng of p24) were mixed and incubated (A) at 37 °C for indicated hours or (B) for 12 h at indicated temperatures. The reaction mixtures were ultracentrifuged to remove intact virions, and p24 amounts in the S_{100} fraction were determined by ELISA.

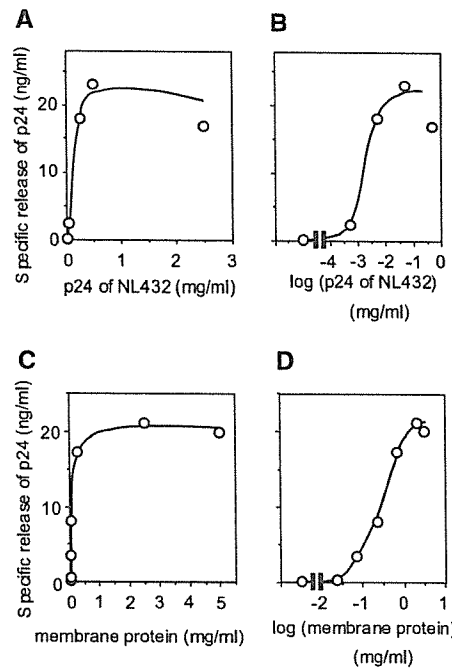

Fig. 4. Requirement of virions and membrane fractions for p24 release in cell-free reaction. Membrane fraction and NL432 virions were mixed in conditions of either (A and B) a fixed membrane amount (10 μg protein) or (C and D) a fixed virion amount (4 ng p24), and were incubated for 12 h at 37 °C. Amounts of p24 in S_{100} were determined by ELISA. Results are expressed in either (A and C) a decimal scale, or (B and D) a semi-logarithmic scale.

saturated after 16–24 h. To examine the temperature dependency of the cell-free p24 release, reaction mixtures were incubated at various temperatures. Fig. 3B demonstrates that p24 was detected at both 37 and 40 °C, but little or scarcely detected at 4 and 25 °C, indicating that the cell-free reaction is temperature dependent.

Specific p24 release was also dependent on the virion and membrane concentrations in the reaction mixtures (Fig. 4). Increasing amounts of virions added to a fixed amount of membrane fraction resulted in increasing p24 release, which was proportional until 250 ng/ml of virions and was saturated at higher concentrations (Fig. 4A, B). Similarly, a fixed amount of virions produced free p24 proportionally to increasing amounts of membrane fraction, which reached a plateau level at more than 0.5 mg/ml of membrane protein (Fig. 4C, D). These results suggest that p24 release in the cell-free reaction reflects specific interaction between the virion and cell membranes.

3.4. Requirement of HIV-1 Env in cell-free p24 release

The involvement of viral Env-gp120 was examined using NL432env(-) virions. Incubation of NL432env(-) with membrane fraction resulted in decreased p24 amounts, when compared with wild-type (wt) NL432 (Fig. 5A). To examine whether endocytic entry activities contribute to the p24 release in the cell-free reaction, NL432env(-) virions were

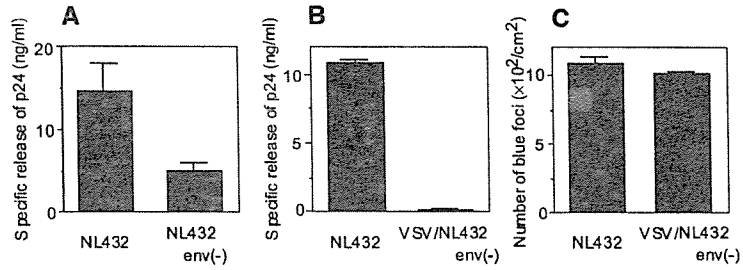

Fig. 5. Env-specificity of p24 release in cell-free reaction. (A) Purified membrane fraction (20 μg protein) from MAGIC5 cells, and NL432 or NL432env(-) virions (4 ng p24) were mixed and incubated for 12 h at 37 °C. Amounts of p24 in S_{100} were measured by ELISA. (B) NL432 and VSV/NL432env(-) pseudotype virions (1.5 ng p24) were mixed with membrane fraction (20 μg protein) and incubated for 27 h at 37 °C. VSV/NL432env(-) was obtained by co-transfection with an equal amount of VSV-G expression vector and NL432env(-) proviral DNA. (C) Infectivity of wt and VSV-pseudotype virions (1.5 ng p24) was determined by blue focus assay.

pseudotyped with the vesicular stomatitis virus G protein (VSV-G) to provide them with the entry ability via the endocytic pathway. Incubation of VSV/NL432env(-) pseudovirions with the membrane fraction resulted in almost undetectable p24 amounts in cell-free assay compared with wt NL432 virions (Fig. 5B), although VSV/NL432env(-) possessed similar levels of infectivity as wt NL432 did (Fig. 5C). These results suggest that p24 release in the cell-free reaction is mediated not by non-specific endocytosis, but rather by the Env-specific fusion/entry pathway of HIV-1.

3.5. Receptor/coreceptor dependence of p24 release

To address the CD4 receptor dependency, membrane fractions were pretreated with anti-CD4 antibodies, which inhibit HIV-1 entry by blockage of the specific gp120-CD4 interaction. As shown in Fig. 6A, anti-CD4 antibody, 7-4, concentration-dependently reduced p24 amounts from NL432 virus. Similarly, pretreatment of membrane proteins (0.5 or 2.5 mg/ml) with another anti-CD4 antibody, Leu 3a, caused a two- to threefold decrease in p24 amounts (Fig. 6B). Specific p24 release from CCR5-utilizing JRFL was also effectively inhibited by pretreatment with anti-CD4 antibody, 7-4 (Fig. 6C).

This CD4-dependent p24 release was examined for its coreceptor specificity. CXCR4-utilizing NL432 produced free p24 by incubation with membrane fraction from CXCR4$^+$ MAGI (CCR5$^-$) and MAGIC5 (CCR5$^+$) cells. In contrast, CCR5-utilizing JRFL induced no significant levels of p24 when incubated with CCR5$^-$ MAGI cell membrane, but induced a high level of p24 when incubated with CCR5$^+$ MAGIC5 cell membrane (Fig. 7A), suggesting that the p24 release reaction reflects coreceptor usage of HIV-1 infection.

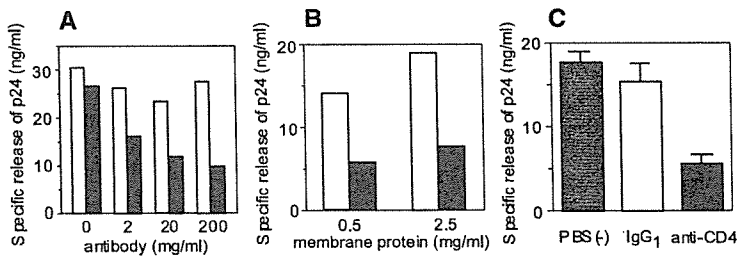

Fig. 6. Inhibitory effect of anti-CD4 on p24 release in cell-free reaction. Fixed protein amounts ((A) 10 μg and (C) 20 μg) or (B) different protein concentrations (0.5 and 2.5 mg/ml) of membrane fraction were preincubated for 30 min with either normal mouse IgG_1 (open bars) or anti-CD4 antibodies (closed bars): (A) increasing concentrations of 7-4, (B) 5 μg/ml of Leu 3a, and (C) 200 μg/ml of 7-4. The treated membrane fraction was then mixed with (A and B) NL432 or (C) JRFL virions (4 ng p24) and incubated for 12 h at 37 °C.

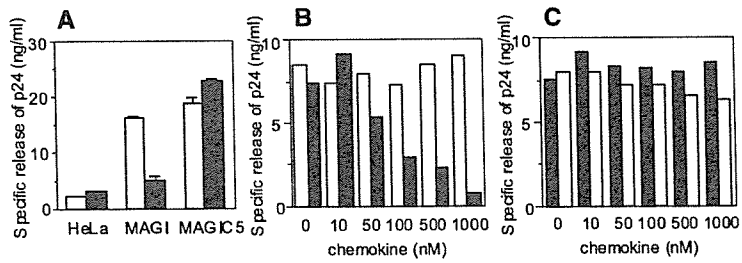

Fig. 7. Coreceptor specificity of p24 release in cell-free reaction. (A) Purified membrane fractions (20 μg protein) from HeLa, MAGI and MAGIC5 cells were incubated with NL432 (open bars) and JRFL (closed bars) virions (3 ng p24) for 27 h at 37 °C. (B and C) Membrane fraction of MAGIC5 cells was preincubated for 30 min with SDF-1α (closed bars) or MIP-1β (open bars), mixed with (B) NL432 or (C) JFRL virions (3 ng p24) and incubated for 12 h at 37 °C.

Therefore, SDF-1α and MIP-1β, which are known to be potent and slight entry blockers of X4 and R5 HIV-1, respectively, were examined to see whether the cell-free reaction is able to distinguish their inhibitory effects. Release of p24 from NL432 was dose-dependently inhibited by SDF-1α but not by MIP-1β, and 1 μM of SDF-1α reduced p24 amounts by 80–90% (Fig. 7B). On the other hand, MIP-1β suppressed p24 release from JRFL by about 20%, but SDF-1α did not (Fig. 7C). These results indicate that the p24 amounts observed in the cell-free reaction were dependent on the CXCR4/CCR5 coreceptors and reflected the inhibitory extents of entry blockers.

4. Discussion

HIV-1 enters into target cells through virus–cell membrane fusion and releases the viral core composed of the p24 capsid protein and RNA genomes [1,2]. This initial step of HIV-1 infection has been examined in most studies using cell cultures, where internalized p24 or viral RNA/DNA was determined by ELISA or PCR in lysates of infected cells [4,5]. Otherwise, the cell-to-cell fusion between HIV-1 Env-expressing cells and appropriate $CD4^+$ target cells has been assayed in cultures [3,6]. A fluorescence dequenching method was also described as a fusion assay where lipid fluorophore was incorporated into the viral membrane before reaction with $CD4^+$ cells [6–8]. A similar method was recently reported using photosensitized labeling of target cells [9]. More recently, a simple and rapid assay has also been developed: cells pre-loaded in cultures with a fluorescent substrate of β-lactamase converted their green emission to blue upon entry of virions incorporated with the enzyme [10,11]. These developments and improvements facilitate sensitive and quantitative assay of HIV-1 fusion/entry.

The next step forward in an approach to the biochemical entry mechanisms may be to develop an in vitro cell-free reaction system that imitates HIV-1 infection. This study attempted to establish a cell-free fusion reaction between HIV-1 and the target cell membrane. To accomplish this, it was necessary to purify both intact infectious HIV-1 particles and membrane fractions of $CD4^+$, $CXCR4^+$ or $CCR5^+$ cells. Our initial attempts using HIV-1-containing culture supernatants and membrane homogenates of target cells failed to provoke significant p24 release from HIV-1 virions in reaction mixtures. As reported previously, culture supernatants of HIV-1-infected or -transfected cells contain a significant fraction of non-infectious virions [32–34], and this is probably responsible for the large variability seen among experiments and for making them difficult to fully recapitulate. Therefore, we purified infectious HIV-1 particles from culture supernatants by ultrafiltration (Fig. 1). Infectious virions thus prepared successfully released p24 into the S_{100} fraction upon reaction to the purified cell membrane in cell-free conditions. The p24 release appears to reflect a specific interaction of HIV-1 with cells, because it occurred in a dose-dependent manner between virion and membrane fractions, in a manner similar to enzyme-substrate reactions (Fig. 4).

HIV-1 entry specifically requires CD4 as receptor and is blocked by some anti-CD4 antibodies [24]. Our cell-free system essentially mimics this CD4 specificity; p24 release in reaction to the $CD4^+$, but not $CD4^-$, cell membrane was dose-dependently inhibited by anti-CD4 antibodies that are known to block HIV-1 entry (Fig. 6). As to coreceptor specificity, CXCR4-dependent NL432 virus entry is known to be efficiently blocked by its ligand, SDF-1α [2,35]. In agreement with the characteristics of NL432 infection, SDF-1α inhibited p24 release of NL432 in our cell-free system, but MIP-1β, a slight entry blocker of CCR5-dependent HIV-1, did not (Fig. 7B). Another JRFL strain of HIV-1 used here requires CCR5 for entry, and our cell-free assay proved that JRFL virions released p24 in reaction to the membrane fraction from $CCR5^+$ cells but not from $CCR5^-$ cells (Fig. 7A), whereas MIP-1β partially inhibited the release (Fig. 7C).

This inefficient inhibition by MIP-1β appears to be consistent with two major modes of the HIV-1 inhibitory action by β-chemokines: direct blocking of Env-coreceptor interaction and indirect interference by downregulation of coreceptors [36–38]. Only 20% of the chemokine effect is estimated to be attributed to direct blocking, and as much as 80% to downregulation [37]. The latter requires cytoplasmic endocytosis factors and thus occurs differentially, depending on the cell types used for analysis. Macrophages and CCR5-transduced adherent cell lines such as HOS-CD4 and HeLa-CD4 cells are reported to be highly unsusceptible with as much as 1 μM of chemokine to prevent HIV-1 entry [38]. Our membrane fraction was prepared from CCR5-transduced HeLa-CD4 cells, removed from cytoplasmic proteins, and its ability to interact with R5 HIV-1 was inhibited by about 20% with 1 μM of MIP-1β. Taken together, these results lead to the conclusion that our cell-free system mimics HIV-1 fusion/entry and therefore is able to detect the efficiency of entry blockers at extents similar to those of culture systems of HIV-1 infection.

Env-mediated fusion reaction requires a higher temperature, whereas HIV-1 binds to target cells at lower temperatures [39,40]. Frey et al. [39] reported that fusion between $CD4^+$ SupT1 or HeLa-CD4 cells with gp160-expressing CHO cells was observed only at temperatures above 25 °C. Similarly, increasing amounts of p24 in cell-free reaction supernatants were dependent on temperature and were induced markedly at temperatures above 25 °C (Fig. 3B). In contrast to the temperature dependence, however, a difference in the time-course was observed between HIV-1 infection and our cell-free p24 release. Fusion reaction of live cells with HIV-1 is usually saturated in 2–3 h [5,9], whereas the p24 release reaction was low after 2 h of incubation, and became saturated at 16–24 h (Fig. 3A).

Although in vitro cell-free reactions are not always equal to those of whole cells in cultures, the slow reaction kinetics may be explained by differences in the receptor/coreceptor