Ultrafree®-MC, Millipore). The filtrate was analyzed by RP-HPLC to determine its solubility.

5.8. Stability of *O*-acyl isopeptides 9–14 in phosphate-buffered saline (pH 7.4)

To 495 μL of PBS (pH 7.4) were added 5 μL of a solution including each *O*-acyl isopeptide (1 mM in DMSO), and the mixture was incubated at 37 °C in a water bath. At the desired time points, 500 μL of 1,1,1,3,3,3-hexafluoro-2-propanol was added to the samples and 500 μL of the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1}, detected at UV 230 nm.

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Perspective

Overcoming Roadblocks in Lead Optimization: A Thermodynamic Perspective

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One of the most important goals in the development of lead compounds is the optimization of their binding affinity towards the intended target, as binding affinity is directly related to potency. Moreover, this goal needs to be accomplished in ways that do not compromise important properties like solubility or bioavailability. Recent experimental and computational developments permit monitoring of the thermodynamic forces that define the binding affinity, the enthalpy and entropy changes, thus providing a rigorous guideline to the optimization process. Having access to individual components of the binding affinity rather than its overall value accelerates the optimization process and facilitates the achievement of extremely high affinity.

Binding affinity originates from different types of interactions between the drug molecule and the target protein as well as their interactions with the solvent (water). In the optimization of binding affinity, there are terms that can be controlled by the designer and terms that are beyond control. The loss of translational degrees of freedom, for example, cannot be altered. For competitive inhibitors that target the same site, the energy associated with conformational changes in the protein is usually constant and cannot be manipulated. The situation is obviously different for drugs that operate allosterically by modulating conformational changes. From the viewpoint of affinity optimization, the binding energy can be viewed as the difference between the interaction energy with the target and the desolvation energy of the drug molecule. These interactions contribute in a characteristic fashion to the enthalpy and entropy of binding, two quantities that can be measured experimentally by isothermal titration calorimetry (ITC) and that can be used to guide the optimization process.

At the thermodynamic level, the binding affinity is determined by the magnitude of the Gibbs energy (ΔG), which is a function of only two terms, the enthalpy (ΔH) and the entropy (ΔS) changes. As the enthalpy and entropy contribute to the binding energy in an additive fashion ($\Delta G = \Delta H - T\Delta S$), it is clear that an infinite number of enthalpy and entropy values can add up to yield the same Gibbs energy value. Compounds that exhibit the same ΔG will bind to the target with the same affinity; however, compounds that are either predominantly enthalpic or entropic will differ in other aspects, as the enthalpy and entropy changes originate from different types of interactions.

The enthalpy change reflects the strength of drug/target interactions in relation to those with the solvent. The favorable term arises primarily from van der Waals and hydrogen bonding interactions between drug and target. Two major terms define the binding entropy; the first one is the solvation entropy associated with the burial from the solvent of hydrophobic groups, and the second one is the conformational entropy, which usually reflects the loss of conformational degrees of freedom upon binding. From the engineering point of view, a favorable enthalpy change is obtained from good geometric complementarity between drug and target and the proper location of hydrogen bond donors and acceptors. As these interactions are stereo-specific, a favorable enthalpy change is not only an important contributor to affinity but also to selectivity. The solvation entropy change reflects a repulsion of the drug from the solvent rather than an attractive interaction with the target. This is a favorable but non-specific force proportional to the hydrophobicity of the drug. The conformational entropy change, on the other hand, usually reflects a loss of conformational degrees of freedom in the drug molecule and protein, being therefore an unfavorable term. The magnitude of the conformational entropy loss can be reduced by introducing conformational constraints in the drug molecule so that it occupies similar conformations in the free and bound states.

Affinity Optimization

The binding affinity of a compound can be improved by generating a favorable binding enthalpy, favorable solvation entropy, or by minimizing the unfavorable conformational entropy. Obviously, extremely high affinity is achieved when the three factors are optimized simultaneously. The degree of difficulty associated with optimizing the enthalpy is not the same as the one associated with optimizing the entropy. Historically, it has proven much easier to optimize the entropy. As the major favorable contributor is the hydrophobic effect, which is proportional to the number of non-polar groups that are buried from the solvent, the tendency throughout the years has been toward an increase in the hydrophobicity of drug candidates (1, 2). Medicinal chemists have long learned to conformationally constrain and pre-shape molecules to the geometry of the binding site, which completes the entropy optimization. According to these

Table 1: Desolvation enthalpy of different chemical functionalities at 25 °C.

Group	ΔH (kcal/mol)	Group	ΔH (kcal/mol)	Group	ΔH (kcal/mol)
NH_2	7.9	OH	8.7	CH_3	0.57
NH	9.4	SO	12.7	CH_2	0.77
N	9.3	CO	5.5	CH	0.73
NO_2	4.7	COO	5.4	$CH_{aromatic}$	0.7
O	5.2	COOH	8.4	C	1.1

Values have been taken from Cabani *et al.* (9) and represent values from water to the gas phase.

traditional precepts, affinity is achieved through hydrophobicity, and selectivity is achieved through shape complementarity.

There is a limit, however, to the hydrophobic character that can be imparted to a compound before it becomes completely insoluble and useless as a drug molecule. At some point in the optimization process, it becomes necessary to introduce favorable enthalpic interactions if the goal is to achieve nanomolar or sub-nanomolar affinities. The exact threshold depends on the characteristics of the target site. Compounds that exhibit extremely high affinity have been shown to display both favorable entropic and enthalpic interactions (3–5). Despite the limits to affinity, a compound that derives selectivity primarily from shape complementarity is prone to lose some when confronted with homologous enzymes with structurally similar binding pockets.

Even though enthalpic interactions are required for extremely high affinity and improved selectivity, the optimization of the binding enthalpy has been notoriously more difficult than the optimization of the binding entropy, the reason being that the enthalpy of desolvation of polar groups is very large and unfavorable, as shown in Table 1. Polar groups carry a desolvation penalty about one order of magnitude larger than non-polar groups. A polar group needs to establish a very good interaction with the target in order to compensate for the desolvation penalty and make a favorable contribution. For this reason, they are often engineered as solubilizers of otherwise extremely hydrophobic compounds rather than major contributors to affinity.

As the major contributors to the binding enthalpy are polar groups, a common misconception is that enthalpically driven compounds must be highly polar and that consequently their bioavailability will be compromised. In fact, what is often observed experimentally is that compounds with the same number of polar groups have vastly different binding enthalpies. For example, among the HIV-1 protease inhibitors, saquinavir and TMC-126 have exactly the same number of polar groups; however, saquinavir binds to the protease with an unfavorable enthalpy of 1.5 kcal/mol, whereas TMC-126 does so with a very favorable binding enthalpy of −12 kcal/mol. To generate a favorable binding enthalpy, it is not the number of polar groups that matters but the quality of their interactions with the target. It is better to have few groups that establish strong interactions than a large number of groups mostly paying the desolvation penalty. In fact, it has been shown that there is no correlation between the enthalpic character of a compound and the Lipinski rules of five (4).

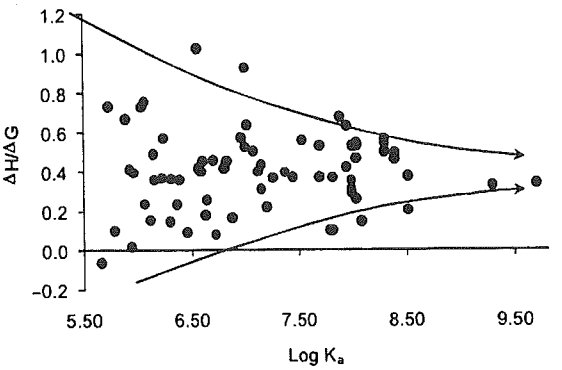

Figure 1: Enthalpic contribution to the Gibbs energy of binding ($\Delta H/\Delta G$) versus the logarithm of the binding affinity (Log K_a) for 71 allophenylnorstatine inhibitors of plasmepsin II. All thermodynamic parameters were determined under identical conditions (10 mM formate buffer, pH 4.0, 2% DMSO, at 25 °C).

Fortunately, the situation is changing on two fronts. Experimentally, ITC permits monitoring of the enthalpy and entropy changes throughout the optimization process and therefore a direct evaluation of the thermodynamic consequences of introducing different functionalities at specific sites. At the computational level, the success of the initial work dealing with the derivation of empirical correlations between binding enthalpy and structural parameters (6) has led to new ways of predicting enthalpy from structure and of predicting the enthalpic effects expected from the introduction of different functionalities into a given scaffold.

The optimization funnel

One of the ongoing projects in this laboratory is the development of plasmepsin inhibitors as new anti-malarial drugs (7). Starting with the allophenylnorstatine scaffold that mimics the main cleavage site in the hemoglobin molecule of infected victims, we have been able to generate high affinity inhibitors with K_i's in the high picomolar range (7, 8). The evolution of the potency of these compounds reflects the situation encountered in most drug discovery laboratories when a given chemical scaffold begins to be optimized. Starting with hits characterized by K_i's in the micromolar range, the goal is to increase potency by three to five orders of magnitude, i.e. an increase in the Gibbs energy of binding of 4–7 kcal/mol. How can this be achieved? How do the individual components of the Gibbs energy advance?

Figure 1 shows the evolution of the contribution of the enthalpy change to the total Gibbs energy of binding as the affinity of the compounds to plasmepsin II is optimized from the micromolar to the high picomolar level. It is immediately apparent that low affinity compounds can exhibit a wide range of enthalpy/entropy combinations. In other words, low affinity can be generated by essentially any combination of hydrophobic and polar interactions. As the affinity increases, the range of enthalpy/entropy combinations narrows

and appears to converge to a smaller range of values as the maximal affinity is approached. For this series of plasmepsin II inhibitors based upon the allophenylnorstatine scaffold, the highest affinity was achieved with a binding enthalpy of −4.5 kcal/mol and an entropic contribution ($-T\Delta S$) of −8.8 kcal/mol.

The data in Figure 1 clearly demonstrates the importance of balancing enthalpic and entropic contributions in order to maximize binding affinity and illustrates important steps in the design process. As enthalpic interactions are more difficult to engineer, enthalpically driven hits are usually easier to optimize than entropically driven ones; i.e. it is less costly energetically to introduce hydrophobic groups. A calorimetric characterization of hits identified by screening or any other method should allow the designer to recognize the nature of the forces by which the hits bind to the target. This step is crucial at these early stages, because it allows separation of those molecules that bind because they are excluded from the solvent from those that bind because they establish favorable interactions with the target. It is at the earlier stages where the spread of enthalpy/entropy combinations is maximal and where a careful decision needs to be made. It is always advantageous to choose compounds that establish good interactions with the target and thermodynamic dissection provides that information. Further down the optimization road, thermodynamic dissection indicates if the process is driven within a reasonable pathway, avoiding thermodynamic extremes that sooner or later lead the process to a roadblock and sometimes a dead end. Obviously, this task is facilitated if it is supplemented by algorithms able to predict the enthalpic or entropic consequences of introducing different chemical functionalities in the scaffold under optimization.

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Original article

Establishment of a biological assay system for human retroviral protease activity

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Abstract

In order to obtain indicator cell lines that are exquisitely susceptible to human T-lymphotropic virus type 1 (HTLV-1), luciferase gene driven by HTLV-1 long terminal repeat (LTR) was transfected into lymphocytic H9 cells with *neo* gene, and cell lines were selected by G418. A cell line (H9/K30*luc*) was found to produce an extremely high level of luciferase only when co-cultured with HTLV-1 producer MT-2 cells. Both in the absence and presence of a reverse transcriptase (RT) inhibitor azidothymidine, H9/K30*luc* cells generated similarly high luciferase activity upon co-cultivation with MT-2 cells. To develop an equivalent system for human immunodeficiency virus type 1 (HIV-1), H9/NL432 cells, which are stably infected with HIV-1 and producing a low level of the virus-like MT-2 cells for HTLV-1, were generated. Together with the indicator cell line H9/H1*luc* for HIV-1 already reported, antiviral effects of some agents on HTLV-1 and HIV-1 could be readily and sensitively evaluated by similar methods. In fact, by using our system, an HIV-1 protease inhibitor, saquinavir, was demonstrated to be highly effective against HIV-1 but not against HTLV-1.

Keywords: HTLV-1; HIV-1; Luciferase; Retroviral protease

1. Introduction

Human T-lymphotropic virus type 1 (HTLV-1), the first well-characterized human retrovirus, causes adult T cell leukemia/lymphoma (ATL) and is associated with several lymphocyte-mediated disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1–4]. ATL is the result of a clonal outgrowth of a CD4-positve T cell that contains integrated, and frequently defective HTLV-1 DNA. HAM/TSP is linked with the immune system-mediated destruction of cells in the spinal cord. Although there is no evidence that expression of the HTLV-1 genome is a significant feature of symptomatic ATL, many studies have strongly suggested that HAM/TSP involves enhanced virus replication as shown by increases in the blood and cerebral spinal fluid of anti-HTLV-1-antibodies, of activated T cells, and of proviral DNA [5–13]. HAM/TSP, therefore, can be treated effectively by reducing the level of replicating HTLV-1 in infected individuals.

Quantitative monitoring of virus infectivity is prerequisite for various basic and clinical studies on viruses. The growth property of HTLV-1 is now difficult to follow in contrast to that of human immunodeficiency virus type 1 (HIV-1), for which various useful assay systems are available [14]. The lack of an eminent quantitative method for HTLV-1 infectivity would be mainly because it grows in cells much more poorly than HIV-1 [15,16], and hampers the systematic analytical study on HTLV-1. We have recently established new indicator cell lines for HIV-1, and have successfully used them to characterize various clones of HIV and simian immunodeficiency virus (SIV) ([14] and unpublished results). By the same strategy, we have established an indicator lymphocytic cell line for HTLV-1 replication carrying luciferase gene as reporter in this study. Our results described here indicated that simply by co-culturing the indicator cells and virus-

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producing cells and by monitoring luciferase activity in the co-cultures, some potential antiviral agents against HTLV-1 and HIV-1 can be examined for their effects.

2. Materials and methods

2.1. Cell cultures, cell viability, and transfection

Cell lines designated 293T [17], H9 [18], H9/H1*luc* [14], MT-2 [19], MT-4 [19], and M8166 [20] were cultured as previously described [21]. An indicator cell line for HTLV-1 designated H9/K30*luc* was established and maintained as reported for the HIV-1 indicator cell line H9/H1*luc* [14]. H9/NL432 cells, which are persistently infected with HIV-1 and producing stably a low level of the virus, were generated by electroporation [21] of an infectious HIV-1 DNA clone pNL432 [21] into H9 cells and culturing them for several months. Cell viability was monitored by the Cell Counting Kit-8 (Wako Pure Chemical Industries Ltd., Osaka, Japan). For transfection of 293T cells, the calcium-phosphate coprecipitation method was used as previously described [21].

2.2. Luciferase and reverse transcriptase (RT) assays

Luciferase activity was determined by the Luciferase Assay System (Promega Co., Madison, WI, USA). RT activity was measured as previously described [22].

2.3. DNA constructs

Expression vectors for the *neo* gene designated pRVSV-neo [23] and HTLV-1 Tax designated pCG-Tax [24] have been previously described. A full-length molecular clone of HTLV-1 designated pK30 was obtained through NIH AIDS Research and Reference Reagent Program (catalog no. 2817). A luciferase reporter vector designated pK30*luc* was constructed by insertion of polymerase chain reaction-amplified entire long terminal repeat (LTR) of pK30 into the *Xho*I and *Hin*dIII sites of pGL3-Basic Vector (Promega Co.).

3. Results

3.1. Establishment and characterization of luciferase system for HTLV-1 infection

Sensitive and quantitative methods to determine HTLV-1 infectivity were currently unavailable. In order to establish indicator cell lines to monitor HTLV-1 infection easily and rapidly, we constructed a reporter clone carrying luciferase gene under the control of HTLV-1 LTR. The resultant construct pK30*luc* was co-transfected with an HTLV-1 Tax expression vector pCG-Tax into 293T cells, and the production level of luciferase was determined. As shown in Fig. 1A, pK30*luc* directed the synthesis of luciferase at a highly enhanced level in response to HTLV-1 Tax. Stable indicator H9 cell lines were selected by co-electroporation of pK30*luc* and pRVSVneo (approximately 10:1 molar ratio) followed by culturing in the presence of G418 (1 mg/ml). As shown in Fig. 1B, out of six clones obtained, clone no.1 generated a high level of luciferase upon co-cultivation with HTLV-1 producer MT-2 cells [19], and was designated H9/K30*luc*. The indicator cell line for HIV-1 designated H9/H1*luc* [14] responded poorly to co-cultivation with MT-2 cells (Fig. 2), which was consistent with the results previously reported [25,26].

We determined whether the observed activation of H9/K30*luc* cells by MT-2 cells can be caused by cell-free HTLV-1 and by newly synthesized HTLV-1 Tax after co-culture. Cell-free virus samples were prepared from various cell cultures including HTLV-1-positive (MT-2), HTLV-1 DNA-positive (MT-4 and M8166), and HTLV-1-negative (H9) cell lines, and inoculated into H9/K30*luc* to monitor luciferase production. As shown in Fig. 3, no evidence for cell-free HTLV-1 infection was obtained. We then examined the effect of azidothymidine (AZT) on the production of luciferase upon co-cultivation of H9/K30*luc* and MT-2 cells. The two cell lines were co-cultured for 48 h in the presence of AZT at various concentrations, and the luciferase activity expressed in the cultures was assayed. As shown in Fig. 4, no

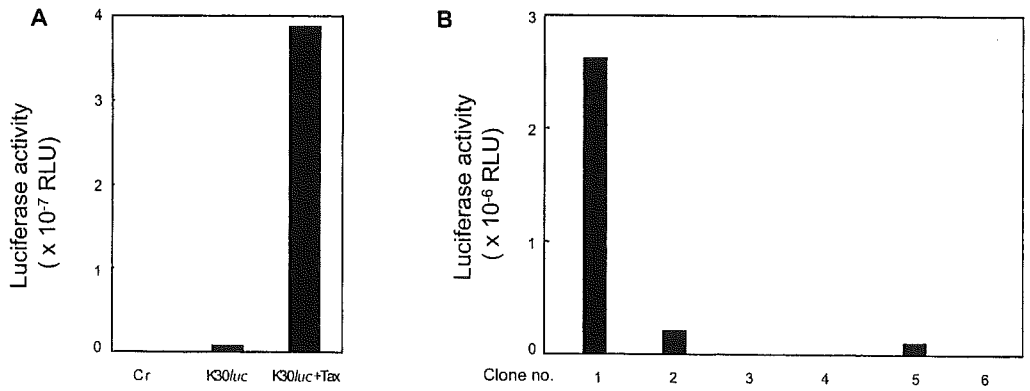

Fig. 1. Activation of HTLV-1 (K30) LTR. (A) Response of the K30*luc* reporter construct to HTLV-1 Tax. 293T cells were co-transfected with pK30*luc* (10 μg) and pUC19 (10 μg) or with pK30*luc* (10 μg) and pCG-Tax (10 μg) as indicated, and 2 days later, cell lysates were prepared for luciferase assay. Control (Cr) 293T cells were singly transfected with 20 μg of pUC19. RLU, relative light unit. (B) Luciferase production in co-cultures of HTLV-1 producer MT-2 cells and H9 cell clones harboring pK30*luc*. G418-resistant H9 cell clones (5×10^5), which had been obtained as described in the text, were co-cultured with MT-2 cells (5×10^5), and on the next day, cell lysates were prepared for luciferase assay.

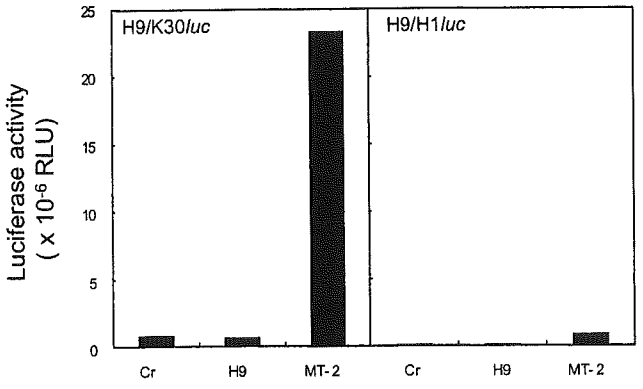

Fig. 2. Enhancement of luciferase production in co-cultures of indicator and MT-2 cells. Indicator cell lines H9/K30*luc* for HTLV-1 (this paper) and H9/H1*luc* for HIV-1 [14] (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6), and 2 days later, cell lysates were prepared for luciferase assay. Cultures of indicator cells only served as Cr.

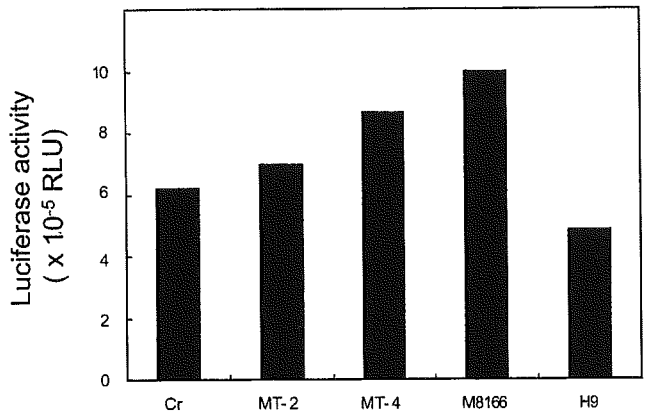

Fig. 3. Potential of cell-free virus from MT-2 to augment luciferase production in H9/K30*luc* cells. Cell-free culture supernatants were prepared from various cultures (MT-2, MT-4, M8166, and H9) maintained at growing phase for 2 days, and inoculated into the indicator cell line H9/K30*luc*. On the next day, cell lysates were prepared for luciferase assay. Culture of indicator cells only served as Cr. Cell lines MT-2 [19] and H9 [18] are HTLV-1-positive and -negative, respectively. Cell lines MT-4 [19] and M8166 [20] are HTLV-1 DNA-positive but negative for HTLV-1.

significant difference was observed among co-cultures of H9/K30*luc* and MT-2 cells.

3.2. Effects of saquinavir (SQV) on HTLV-1 and HIV-1 as determined by our luciferase system

Based on the results described above, we assumed that Tax transported from MT-2 to H9/K30*luc* cells by Env-mediated membrane fusion enhances the luciferase production, and that, if this process is suppressed, luciferase production is significantly reduced. It has been reported for HIV-1 recently that interactions between unprocessed Gag and the cytoplasmic tail of Env-gp41 suppress cell fusion [27]. We, therefore, checked by our system the effects of a protease inhibitor SQV on HIV-1 and HTLV-1. SQV has been reported to be very effective against HIV-1 protease but fails to inhibit HTLV-I Gag processing in infected cells [28]. To obtain appropriate HIV-1 producer cells, which are stably infected with HIV-

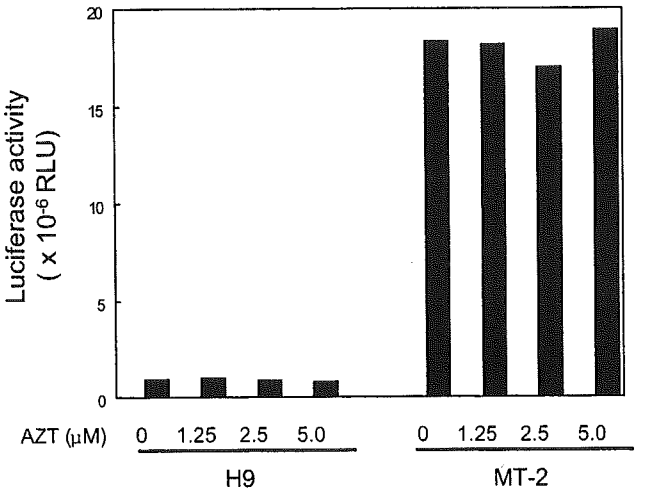

Fig. 4. Effect of AZT on luciferase production in the co-cultures of H9/K30*luc* and MT-2 cells. Indicator H9/K30*luc* cells (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6) in the absence or presence of AZT as indicated for 48 h, and cell lysates were prepared for luciferase assay. H9 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of AZT as above before co-culture started. No cytotoxic effects were observed in these conditions.

1 and producing a low level of the virus-like MT-2 cells for HTLV-1, H9 cells were electroporated with pNL432 and cultured for months. The resultant H9/NL432 cells were easily maintained, and produced a low level of HIV-1 as monitored by RT assay (data not shown).

By the use of H9/H1*luc*, H9/K30*luc*, H9/NL432, and MT-2 cells as indicator and virus producer cells, we determined the inhibitory effects of SQV on HIV-1 and HTLV-1 by monitoring luciferase activity. The effects of SQV on viability of cells were also determined to confirm that there would be no experimental error caused by cytotoxicity. As shown in Fig. 5, while luciferase production in the HIV-1 co-culture was severely inhibited by SQV, no appreciable effects were observed for the HTLV-1 co-culture. These data were in good agreement with our assumption and the results previously reported [27,28] as mentioned above.

4. Discussion

In this report, we have established an indicator cell line for HTLV-1 infection based on luciferase assay (Figs. 1 and 2). Although the cell line H9/K30*luc* was highly susceptible to infection by the co-culture method (Fig. 1), it was insensitive to infection with cell-free HTLV-1 (Fig. 3) as expected [15,16]. Enhanced production of luciferase observed in the co-cultures of H9/K30*luc* and MT-2 cells was probably due to the Tax already present in MT-2 cells before co-cultivation (Fig. 4). Therefore, we have concluded that our HTLV-1 system described here monitors the efficiency of Env-mediated membrane fusion, and that it is useful for evaluating the ability of various factors or agents affecting the process. Indeed, SQV was demonstrated to be a powerful inhibitor for HIV-1 by affecting cell fusion indirectly (Fig. 5).

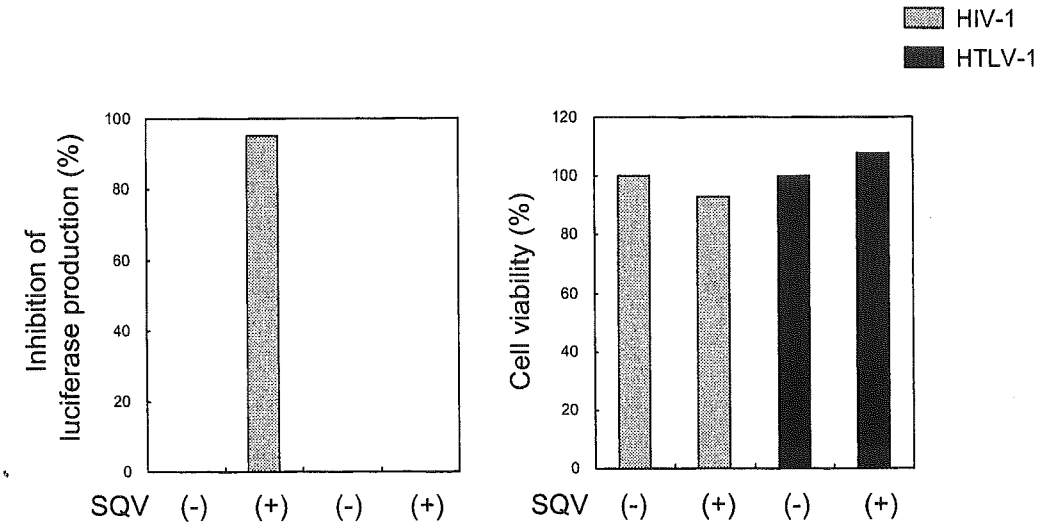

Fig. 5. Effects of SQV on the co-cultures of indicator and virus-producing cells. Indicator (H9/H1*luc* for HIV-1 and H9/K30*luc* for HTLV-1) and virus-producing cells (H9/NL432 for HIV-1 and MT-2 for HTLV-1) (10^6 for each) were co-cultured for 48 h in the absence (–) or presence of SQV (+, 2 μM), and cell lysates were prepared for luciferase assay. Data presented are relative to those of cultures without SQV. H9/NL432 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of SQV as above before co-culture started. Cell viability was determined by the Cell Counting Kit-8, and relative values are presented.

Our results described here strongly suggest that our biological assay system can be used for screening of inhibitors against HTLV-1 and HIV-1 proteases. In particular, because HIV-1 protease inhibitors cannot be effective against HTLV-1 protease (Fig. 5) [28], and because no other good assay methods are available for HTLV-1, the screening by the H9/K30*luc*-MT-2 system would be important. Furthermore, pathogenesis of HAM/TSP can be controlled by reducing the level of HTLV-1 in infected individuals [5–13].

Our protocol for monitoring the inhibitory effects of potential protease inhibitors on HTLV-1 and HIV-1 is summarized as shown in Fig. 6. By using appropriate producer and indicator cells, a large number of antiviral agents can be checked readily for their ability to inhibit the replication of HTLV-1 and HIV-1 within days. Screening of various candidate protease inhibitors by the protocol in Fig. 6 is now in progress in our laboratory.

Virus-producer cells
↓
Culture in the presence of protease inhibitors for 24hrs
↓
Co-cultivation with indicator cells in the presence of protease inhibitors for 48hrs
↓
Assays for luciferase and cell viability

Virus	Producer cells	Indicator cells
HIV-1	H9/NL432	H9/H1*luc*
HTLV-1	MT-2	H9/K30*luc*

Fig. 6. Evaluation system for the effects of human retroviral protease inhibitors. Based on the results in this report, validity of human retroviral protease inhibitors can be readily evaluated as shown in this figure.

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Original article

Unique characteristics of HIV-1 Vif expression

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Abstract

We examined the steady-state expression in cells of four accessory proteins of human immunodeficiency virus type 1 (HIV-1). For this purpose, a series of single gene expression vectors for these viral proteins were constructed and were monitored for their production by transfection. Among them, the expression level of Vif was found to be lowest in both the absence and presence of APOBEC3G. In addition, we noticed the presence of its truncated form, which was not observed for the other accessory proteins. When a subgenomic vector was used for transfection, authentic and several small forms of Vif were produced. By mutational analysis, these forms were demonstrated to be mutant Vif proteins translated from M^{8}, M^{16} and M^{29}. When a full-length molecular clone was used, the smaller versions of Vif were hardly observed. Functional analysis of these mutant Vif proteins showed that they are incapable of modulating viral infectivity. The results described above, i.e. the low steady-state expression and the presence of truncated forms, represent the unique characteristics of HIV-1 Vif.

Keywords: HIV-1; Vif; APOBEC3G; Accessory protein

1. Introduction

Four accessory proteins of human immunodeficiency virus type 1 (HIV-1) are known to modulate viral infectivity and various functions of target cells [1]. Vif, one of these proteins, is encoded by the gene that is conserved in all known primate immunodeficiency viruses [2]. It has a critical role in conferring infectivity on progeny virions in a producer cell-dependent manner [3–6]. Virions produced in non-permissive cells, such as primary cells of lymphocytic or monocytic origin and a limited number of cell lines like H9, are unable to replicate in any kinds of target cells. Recent works have demonstrated that Vif counteracts anti-viral activity of human cytidine deaminase APOBEC3G present in non-permissive cells [7–15]. The precise molecular mechanism for this activity of Vif, however, is still controversial [16] and remains to be elucidated.

We have previously shown that Vif is rapidly decayed in cells in both the absence and presence of APOBEC3G, and that the expression of Vif to an excessive level is inhibitory to viral replication [17]. The fragile nature of Vif was unique among the accessory proteins and was mediated, at least in part, by the host proteasome system [18]. In the present report, to determine whether the expression of Vif is maintained to be uniquely low in cells in the absence of the other viral factors, the four accessory genes of HIV-1 were separately cloned into expression vector with tag, and examined for their steady-state expression level. During the course of this comparative study, we detected a truncated form of Vif, but no extra versions were observed for the other accessory proteins. We, therefore, examined the expression pattern of Vif from a subgenomic clone, and found two other small forms. Mutational and functional analyses of the three small Vif proteins were also carried out in this study.

2. Materials and methods

2.1. Plasmids

2.1.1. Full-length molecular clones

A full-length molecular clone pNL432 (GenBank Accession no. AF324493) was used for production of wild-type

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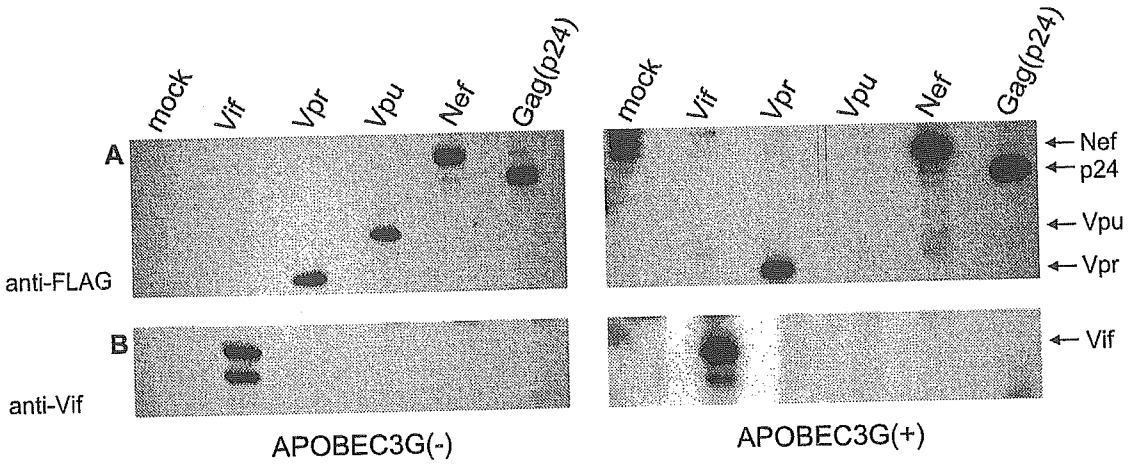

Fig. 1. Production of HIV-1 (NL432) accessory proteins by expression vector pSG-FLAG. 293T cells were transfected with 10 μg of pSG-FLAG vectors with (APOBEC3G(+)) or without (APOBEC3G(–)) 1 μg of pcDNA-APO3G (expression vector for APOBEC3G) [9]. Cells were lysed in Laemmli sample buffer at 48 h post-transfection for Western blot analysis. Each lane contained 50 ng of protein. The Abs used here were anti-FLAG M2 monoclonal Ab (panels A) and anti-Vif peptide Ab [22] (panels B). An expression vector for Gag-p24, designated pSG-Gag (p24) cFLAG [18], was used for control. Mock, pSG5.

(wt) infectious virus [19]. Frame-shift mutants of pNL432, designated pNL-Nd [20,21], pNL-Kp [20] and pNL-NdKp [22], were used for production of *vif*-minus, *env*-minus and *vif/env* double-minus viruses, respectively.

2.1.2. Subgenomic vectors

A subgenomic expression vector, designated pNL-A1S, was constructed from pNL-A1 [23]. For easy insertion of DNA fragments from pNL432 [19], it has a unique *Sma*I site (Fig. 2B) newly generated by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Various subgenomic expression vectors for wt and mutant Vif proteins described below were constructed by insertion of PCR-amplified *vif* sequences having *Sma*I and *Eco*RI sites at 5′ and 3′ ends, respectively, into pNL-A1S (Fig. 2B). PCR templates for construction of pNL-A1S-fWT, pNL-A1S-Nd, pNL-A1S-fM16A, pNL-A1S-fM8/16A and pNL-A1S-fM8/16/29A were pNL432 [19], pNL-Nd [20,21], pNL-fM16A, pNL-fM8/16A and pNL-fM8/16/29A, respectively. Clones pNL-fM16A, pNL-fM8/16A, and pNL-fM8/16/29A are mutants of pNL432 carrying M16A, M8/16A and M8/16/29A mutations, respectively (Fig. 3A).

2.1.3. Expression vectors for a single gene

Vectors pcDNA-APO3G [9] and pCMV-G [24] were used to express APOBEC3G and vesicular stomatitis virus G protein (VSV-G), respectively. Expression vectors for wt and mutant Vif proteins, designated pSG-Vif, pSG-fΔ7, pSG-fΔ15 and pSG-fΔ28, were constructed by insertion of PCR-amplified *vif* sequences having *Eco*RI and *Bam*HI sites at 5′ and 3′ ends, respectively, into pSG5 (Stratagene). As template for PCR, pNL432 [19] was used. An expression vector for wt Vif tagged with FLAG at the C-terminus, designated pSG-Vif cFLAG, was constructed in the same way as for pSG-Gag (p24) cFLAG [18]. Expression vectors for the other accessory proteins tagged with FLAG at the C-terminus, designated pSG-Vpr cFLAG, pSG-Vpu cFLAG and pSG-Nef

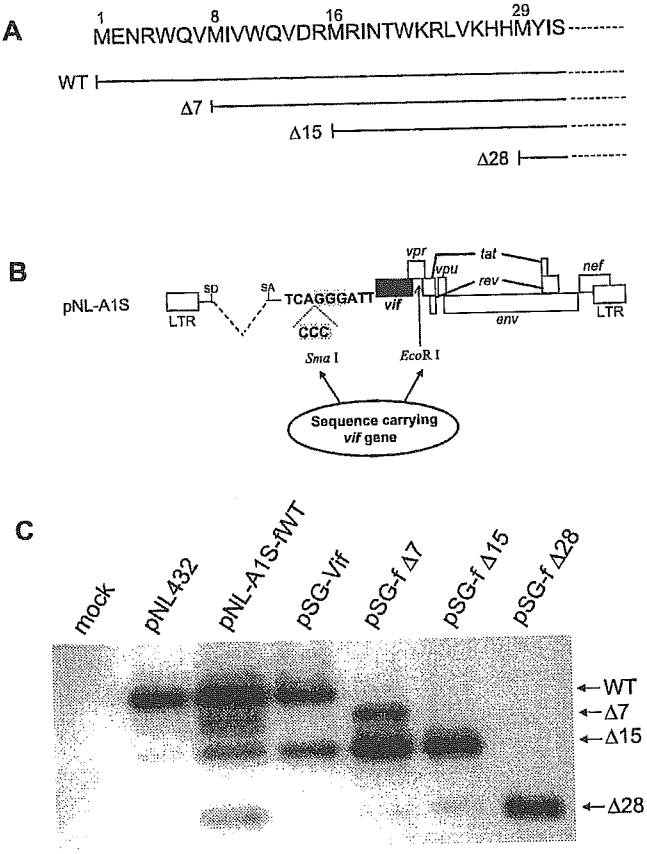

Fig. 2. Expression of HIV-1 (NL432) Vif-related proteins by various clones. (A) Structure of three putative mutants of Vif. Mutants Δ7, Δ15, and Δ28 lack N-terminal 7, 15 and 28 amino acids, respectively. (B) Structure of expression vector for Vif (pNL-A1S). Vector pNL-A1S has a new and unique *Sma*I site (relative to parental clone pNL-A1) for cloning, as indicated. (C) Monitoring of Vif-related proteins by Western blot analysis. 293T cells were transfected with 10 μg of various vectors, as indicated, and cell lysates were prepared by CHAPS buffer at 48 h post-transfection. Anti-Vif peptide Ab was used for detection as previously described [22]. Each lane contained 50 ng of protein, execpt for the lane for pNL-A1S-fWT (5 ng of protein). Mock, pUC19.

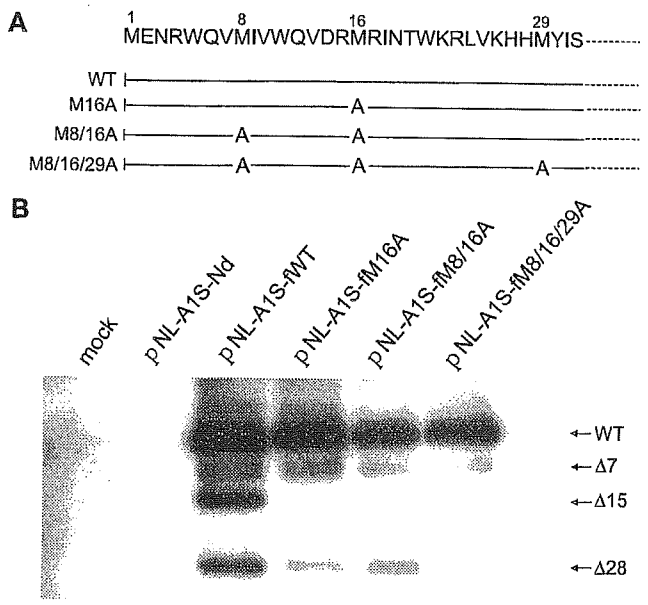

Fig. 3. Expression of various species of HIV-1 (NL432) Vif proteins by subgenomic pNL-A1S constructs. (A) N-terminal amino acid sequence of mutant Vif proteins. Mutant clones have alanine residues instead of methionines, as indicated. (B) Monitoring of Vif-related proteins expressed by subgenomic pNL-A1S constructs. 293T cells were transfected with 10 μg of various vectors, as indicated, and cell lysates were prepared in Laemmli sample buffer at 48 h post-transfection. Anti-Vif peptide Ab was used for detection, as previously described [22]. Each lane contained 5 ng of protein. Mock, pUC19.

cFLAG, were constructed by replacement of the *gag* gene of pSG-Gag (p24) cFLAG [18] with PCR-amplified accessory gene sequences having *Eco*RI and *Xho*I sites at 5′ and 3′ ends, respectively. As template for PCR, pNL432 [19] was used.

2.2. Cells

H9 [25] and 293T [26] cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS, respectively. MAGI cells [27] were cultured as for 293T cells in the presence of 0.2 mg/ml of G418 and 0.1 mg/ml of hygromycin B.

2.3. Transfection and reverse transcriptase (RT) assay

For transfection of 293T cells, the calcium-phosphate coprecipitation method was used as previously described [19]. Virus production in the culture supernatants was monitored by RT activity as previously described [28].

2.4. Western immunoblotting

Cell lysates for Western blotting analysis were prepared by CHAPS/DOC [28] or Laemmli's sample buffer [18] as previously described. Samples were resolved by SDS-PAGE followed by electrophoretic transfer to polyvinylidene fluoride membranes. The membranes were treated with anti-FLAG M2 monoclonal antibody (Ab) (SIGMA-ALDRICH Corp., St. Louis, MO, USA) or anti-Vif peptide Ab [22], and visualized with the ECL plus Western Blotting Detection System (Amersham Biosciences UK Limited, Buckinghamshire, UK) as previously described [29,30].

2.5. MAGI assay

To determine the infectivity of virions, MAGI assay was performed as previously described [27].

3. Results

3.1. Low expression level of Vif protein in cells

Recent work by ourselves has shown that Vif expressed from viral full-genome is degraded rapidly and maintained to be at low level in cells by proteasome [18], and that this unstable nature of Vif was unique among the accessory proteins [18]. We asked, in a quantitative and comparable manner, whether the expression level of Vif is uniquely low in cells in the absence of the other viral proteins. For this purpose, the four HIV-1 accessory genes, *vif*, *vpr*, *vpu* and *nef*, were separately cloned into expression vector pSG5-FLAG tag, and four expression vectors for each accessory protein, designated pSG-Vif cFLAG, pSG-Vpr cFLAG, pSG-Vpu cFLAG and pSG-Nef cFLAG were constructed. These clones were then transfected into permissive 293T cells with or without APOBEC3G expression vector pcDNA-APO3G [9] and monitored for their expression by Western blot analysis using anti-FLAG antibody. Laemmli's sample buffer was used to prepare cell lysates for analysis, since Vif was quite refractory to CHAPS/DOC buffer [28,31]. As shown in Fig. 1A, regardless of the presence or absence of APOBEC3G, no Vif was detected, in contrast to the results for Vpr and Nef, when anti-FLAG Ab was used. Worthy of note, in the presence of APOBEC3G, Vpu was not detected at all. In the absence of APOBEC3G, Vpu was expressed at a level similar to that of Vpr. We wished to examine the expression of Vif using Ab other than anti-FLAG Ab. As shown in Fig. 1B, when the same blots in Fig. 1A were re-probed with anti-Vif peptide Ab, two Vif-related bands (~23 and ~21 kDa) appeared both in the absence and presence of APOBEC3G. These probably represented authentic and truncated forms of Vif.

The results described here indicated that Vif is certainly expressed in cells at a relatively low level. Another important finding was the presence of the truncated version of Vif.

3.2. Truncated forms of Vif proteins

We assumed that, on the basis of its size, the truncated form of Vif is a mutant Vif translated from M^{16}, as shown in Fig. 2A. To verify this hypothesis, expression vectors of wt Vif and its mutants, Δ7, Δ15 and Δ28, based on pSG5 (pSG-Vif, pSG-fΔ7, pSG-fΔ15 and pSG-fΔ28), were constructed (Fig. 2A) and used for monitoring of their products in cells upon transfection. A subgenomic expression vector, desig-

nated pNL-A1S-fWT, was also constructed (Fig. 2B) and used, because the expression level of Vif by this vector was expected to be very high [23]. The vectors constructed were then introduced into 293T cells and examined for the expression pattern of Vif by Western blotting technique. As shown in Fig. 2C, while wt full-length clone pNL432 generated a distinct and single Vif, the subgenomic clone pNL-A1S-fWT produced several Vif proteins recognizable by anti-Vif peptide Ab. The mutant pSG constructs expressed Vif-related proteins well-anticipated by our assumption. It was, therefore, not unreasonable to conclude that there are three mutant Vif proteins starting from M^8, M^{16} and M^{29}. To prove this, three methionines present in the N-terminal region of Vif (Fig. 3A) were changed to alanines, and the mutants constructed were examined for their products upon transfection (pNL-A1S series in Fig. 3B). The results obtained were in good agreement with our prediction. Mutants pNL-A1S-fM16A, pNL-A1S-fM8/16A and pNL-A1S-fM8/16/29A did not produce a central major band (Δ15). Mutant pNL-A1S-fM8/16/29A did not express the smallest band (Δ28) at all. In addition, one of the faint bands (Δ7) just below the authentic Vif appeared to disappear for mutants pNL-A1S-fM8/16A and pNL-A1S-fM8/16/29A. The data described above were reproduced for the samples prepared in the presence of APOBEC3G (data not shown). Furthermore, consistently with the data described above, the M16A mutant of pSG-construct did not produce the major Δ15 band upon transfection (data not shown).

3.3. Biological activity of small versions of Vif proteins

We asked ourselves whether truncated forms of Vif were able to confer infectivity on progeny virions. *Vif*-minus full-length clone pNL-Nd [20,21] and expression vectors of Vif or its mutants (pSG-Vif, pSG-fΔ7, pSG-fΔ15 and pSG-fΔ28) with or without pcDNA-APO3G [9] were co-transfected into 293T cells, and the effects of various Vif pro-

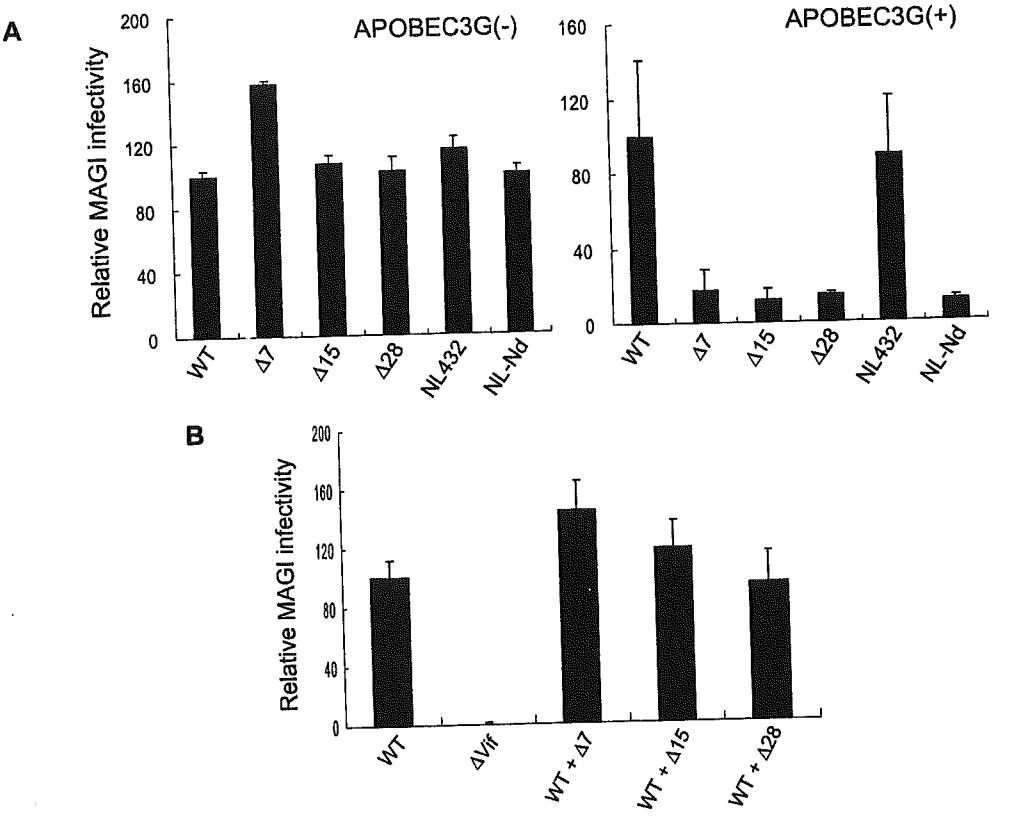

Fig. 4. Biological evaluation of various species of HIV-1 (NL432) Vif. (A) Ability of mutant Vif proteins to confer infectivity on progeny virions. 293T cells were triply transfected with 2.5 μg of pNL-Nd [20,21], 7.5 μg of expression vector (pSG-Vif (WT), pSG-fΔ7, pSG-fΔ15 or pSG-fΔ28) and 1 μg of pcDNA-APO3G (expression vector of APOBEC3G) [9] (APOBEC3G(+)), or dually transfected with the former two clones (APOBEC3G(–)). At 48 h post-transfection, culture supernatants were harvested for virus samples as previously described [29]. As control for virus samples, culture supernatants prepared from 293T cells transfected with wt pNL432 [19] or ΔVif mutant pNL-Nd [20,21] with (APOBEC3G(+)) or without pcDNA-APO3G [9] (APOBEC3G(–)) were used. The prepared virus samples were inoculated into MAGI cells, and infectivity was determined as previously described [27]. MAGI infectivity was normalized by RT activity and shown as relative values. (B) Effects of mutant Vif proteins on viral infectivity. 293T cells were co-transfected with 1.3 μg of *env*-minus full-length clone pNL-Kp [20], 1.3 μg of pCMV-G (expression vector of VSV-G) [24], 7.5 μg of expression vector (empty vector pSG5, pSG-fΔ7, pSG-fΔ15 or pSG-fΔ28), and 1 μg of pcDNA-APO3G (expression vector of APOBEC3G). Under these conditions, similar amounts of wt Vif and Δ15 mutant were expressed from pNL-Kp and pSG-fΔ15, respectively. Virus samples were harvested at 48 h post-transfection, and their infectivity was determined by MAGI assay, as described in (A). MAGI infectivity was normalized by RT activity and shown as relative values. WT, pNL-Kp + pSG5; ΔVif, pNL-NdKp + pSG5; WT + Δ7, pNL-Kp + pSGfΔ7; WT + Δ15, pNL-Kp + pSGfΔ15; WT + Δ28, pNL-Kp + pSGfΔ28. A full-length clone pNL-NdKp [22] was *vif/env* double-minus, and served as a negative control for pNL-Kp [20].

teins on viral infectivity were examined. As shown in Fig. 4A, in the absence of APOBEC3G, all virus samples showed similar MAGI infectivity, as expected. In the presence of APOBEC3G, while wt Vif conferred an infectivity on virions comparable to that of wt virus NL432, none of the truncated forms of Vif did like ΔVif mutant virus NL-Nd. We then examined whether truncated forms of Vif had negative effects on infectivity of virions. *Env*-minus full-length clone pNL-Kp [20], one of the expression vectors for Vif mutants as above, an expression vector of VSV-G protein pCMV-G [24] and pcDNA-APO3G [9] were co-transfected into 293T cells, and the infectivity of viruses produced at 2 days post-transfection was determined. As shown in Fig. 4B, no truncated forms of Vif exhibited any significant negative effects on viral infectivity.

4. Discussion

One of the major findings in this study is that the expression of Vif is consistently controlled to be low (Fig. 1). We have previously proposed a mechanism for this observation, that is, proteasome degradation [18]. Vif was much more rapidly degraded than any other accessory proteins and kept low in cells. The other possible explanation for the restricted expression of Vif is related to mRNA. Expression of mRNA for Vif was reported to be maintained to be limited [32]. The unstabilness of mRNA for Vpu was also reported [32], and this could cause the lower expression level of Vpu compared with that of Nef (Fig. 1). In this context, the lack of detectable expression of Vpu in the presence of APOBEC3G (Fig. 1) was intriguing. The plausible mechanism for the down-regulation of Vpu could be the introduction of mutations to mRNA for Vpu by the cytidine deaminase activity of APOBEC3G and/or the enhancement of degradation of Vpu by APOBEC3G.

Another major finding in this report is the production of truncated forms of Vif (Figs. 1–3). We showed evidence here by mutational analysis that these Vif-related proteins are translated from M^8, M^{16} or M^{29} (Fig. 3). To the best of our knowledge, this is the first report that shows the initiation of translation of HIV-1 proteins at a methionine codon other than M^1. Of note, the expression pattern of the Vif proteins varied depending on the clones used. Clones pSG-Vif (single gene) and pNL-A1S-fWT (subgenome) expressed one (Δ15) and three (Δ7, Δ15 and Δ28) Vif proteins, respectively (Figs. 1–3). Full-genomic pNL432 appeared not to express any small forms of Vif (Fig. 2). The molecular basis for this observation remains to be elucidated. It is also important to determine whether the truncated Vif proteins described here are biologically active. Our functional analysis in this report showed that small forms of Vif are biologically inactive (Fig. 4). It is still possible, however that these Vif proteins affect the replication of virus by unknown mechanisms.

Acknowledgements

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遺伝子診療学

—遺伝子診断の進歩と遺伝子治療の展望—

A. 遺伝子診断（genetic diagnosis）

（遺伝学的検査 genetic testing，遺伝子検査 gene-based testing，
核酸検査 nucleic acid-based testing）

V. 感染症の核酸検査（nucleic acid-based testing）

HIV感染症

足立昭夫

A. 遺伝子診断(genetic diagnosis)

(遺伝学的検査 genetic testing, 遺伝子検査 gene-based testing, 核酸検査 nucleic acid-based testing)

V. 感染症の核酸検査(nucleic acid-based testing)

HIV感染症

HIV infection

足立昭夫

Key words：エイズ，HIV-1，遺伝子診断，遺伝子治療

はじめに

エイズの原因ウイルスであるヒト免疫不全ウイルス1型(human immunodeficiency virus type 1: HIV-1)は1983年に発見された．1986年には第二のヒトエイズウイルス(HIV-2)も同定されたが，世界中に蔓延し人類の脅威となっているのはHIV-1である．アジアやアフリカ地域においては現在もなおHIV-1感染者は非常な勢いで増加し続けている．しかし，この20年間でHIV基礎研究は飛躍的な発展を遂げた．ウイルスゲノムやウイルス蛋白質は詳細に解析され，細胞レベルでのウイルス複製機構は大筋で解明された[1,2]．ウイルスの進化や伝播に関する情報も蓄積され，エイズの予防・治療に密接に関連する分子疫学的研究も進展した[3,4]．現在は，これらを有機的に統合し基礎と臨床をリンクさせた研究が主流となっている．

本稿では，基礎/臨床研究の成果に基づくHIV-1感染の遺伝子診断およびエイズの遺伝子治療について現状を概説する．

1. 概　　念

HIV-1はレトロウイルス科に属するので，基本的にこのウイルス群特有の複製様式に従って増殖する(**図1**)．ただし，単純なレトロウイルスよりはるかに多い9種の遺伝子をもつため，レンチウイルス属固有の特徴も有している(**表1**)．個体内でのウイルス増殖は極めて緩やかであるが，次第に体内ウイルス量が上昇し，感染者は徐々にエイズへと進行していく．HIV-1の遺伝子診断や遺伝子治療はこれらの事実に基づいて成立している．

2. 歴史的考察

病原ウイルス感染の有無は，ウイルス分離，ウイルス抗原や核酸の証明および血清学的検査などにより判断される(**表2**)．ウイルス学あるいは免疫学的手技を用いたウイルス検査・診断は以前から広く行われてきた．検査・診断手法を選択する場合，特異性，迅速性，簡便性，再現性，感度などが重要であり，対象とするウイルスの性質により適切なものが異なることは言うまでもない．HIV-1はその発見直後からゲノム研究が精力的かつ広範囲にわたって行われたので，早い時期から遺伝子診断や遺伝子治療が可能となっていた．

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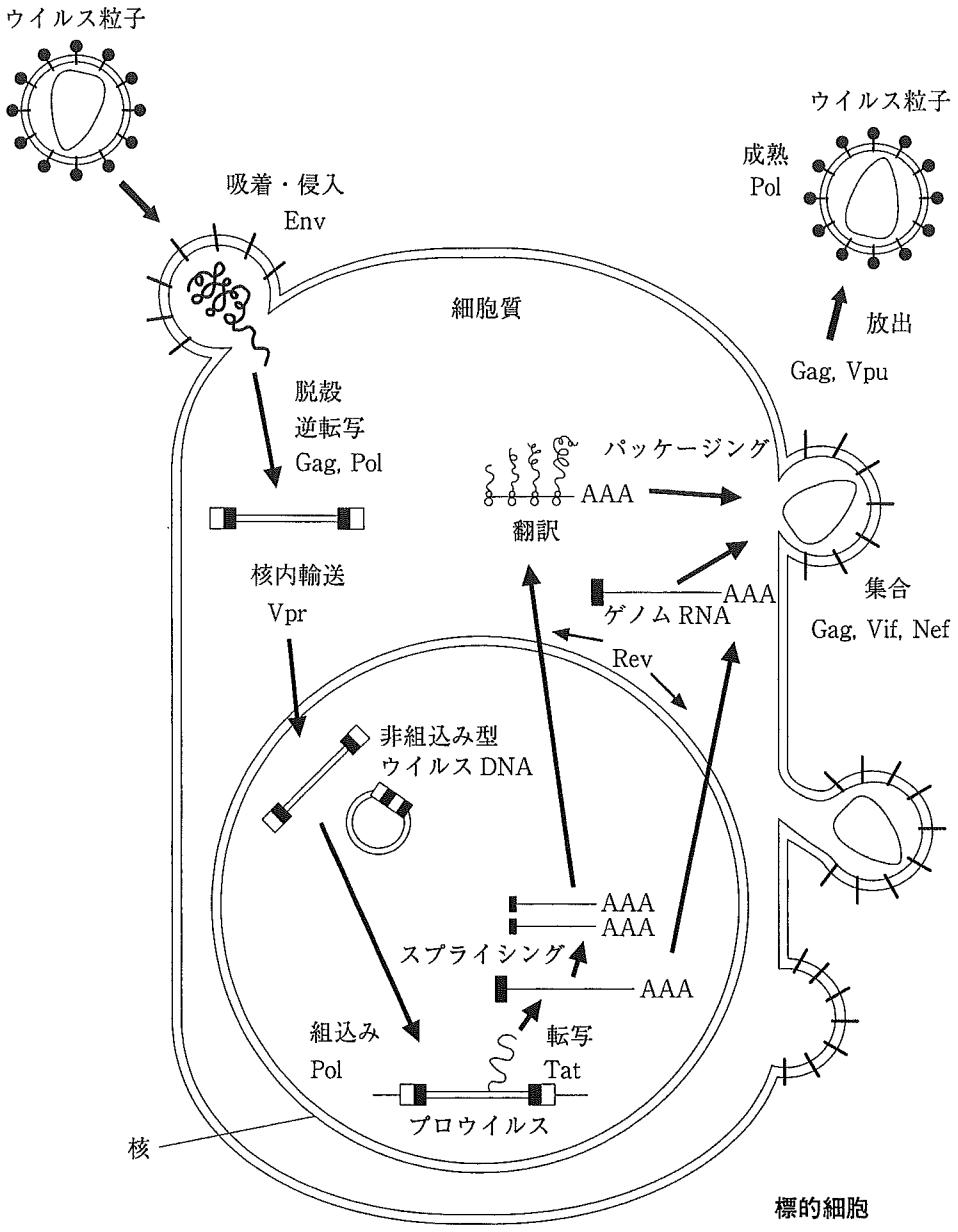

図1 HIV-1の複製サイクル

標的細胞におけるHIV-1複製の各ステップとそれに関与するウイルス蛋白質(**表1**)を示す．主な標的細胞はCD4陽性Tリンパ球およびマクロファージである．(文献[2]から一部改変して引用)．

3. 遺伝子診断・遺伝子治療

a. 遺伝子診断

HIV-1感染のスクリーニングは，通常抗ウイルス抗体の検出により行われる．迅速検査のための簡便な検査キットも市販されているので，ウェスタンブロット(WB)法による確認検査を含めても短時間でウイルス感染の有無が判定できる[5]．核酸増幅検査(nucleic acid amplification test: NAT)はポリメラーゼ連鎖反応(polymerase chain reaction: PCR)の原理に基づくもので，非常に検出感度が良くかつ定量も容易である[5]．このシステムはHIV-1感染症の検査・診断法として確立している．市販のRT(reverse transcription)-PCRキットにより，血中ウイルス量が測定でき，診断だけでなく個体内のウイルス動態も解析可能となっている．RT-PCR法を用いれば，薬剤抵抗性変異ウイルスの検出も容易

表1 HIV-1蛋白質とその主要な機能

ウイルス蛋白質		機 能
構造蛋白質		
Gag	MA(マトリックス)	ウイルス粒子形成・放出，脱殻・逆転写
	CA(カプシド)	ウイルス粒子形成・放出，脱殻・逆転写
	NC(ヌクレオカプシド)	脱殻・逆転写
	p6	ウイルス粒子放出，Vpr/Vpxのウイルス粒子へのターゲッティング
Pol	PR(プロテアーゼ)	Gag前駆体やGag-Pol前駆体の開裂による成熟ウイルス粒子(感染性ウイルス粒子)の生成
	RT(逆転写酵素)	ウイルスDNA合成(含RNase H活性)
	IN(インテグラーゼ)	ウイルスDNAの細胞染色体DNAへの組込み
Env	gp120	標的細胞受容体への結合
	gp41	標的細胞への侵入
調節蛋白質		
Tat		転写の増強
Rev		構造およびアクセサリー蛋白質(Nefを除く)の発現増強
アクセサリー蛋白質		
Vif		抗ウイルス細胞因子APOBEC3Gの不活化
Vpr		ウイルスDNAの核移行
Vpu		ウイルス粒子の放出促進
Nef		細胞表面におけるMHC-I発現の抑制

表2 病原ウイルスの検査・診断法

検出対象	方 法
感染性ウイルス	培養細胞などへ接種しウイルスを分離
感染細胞	細胞変性効果の観察など
ウイルス粒子	電子顕微鏡による形態の観察，赤血球凝集能測定，特異酵素検出など
ウイルス抗原	蛍光抗体法，ELISA(enzyme-linked immunosorbent assay)，RIA(radioimmunoassay)などによる特異抗原検出
ウイルス核酸	PCR(polymerase chain reaction)あるいはRT-PCR(reverse transcription-PCR)などによる特異核酸の検出
抗ウイルス抗体	ELISA，CF(complement fixation)，HI(hemagglutination inhibition)，WB(Western blot)などによる特異抗体の検出

である．

b. 遺伝子治療

HIV-1感染者の治療法として現在最も有効なものは多剤併用療法(highly active anti-retroviral therapy: HAART)である[6,7]．感染者の体内ウイルスの動態をモニターしつつHAARTを行うことで大きな成果を上げている．しかし，最近の研究から，HAART導入後も潜伏持続感染細胞が減少しないなどウイルス増殖は完全には抑え込めないこと，また，薬剤抵抗性ウイルスの出現も明らかになってきた[6,7]．このため，HIV-1の遺伝子治療は全く新しい治療戦略と