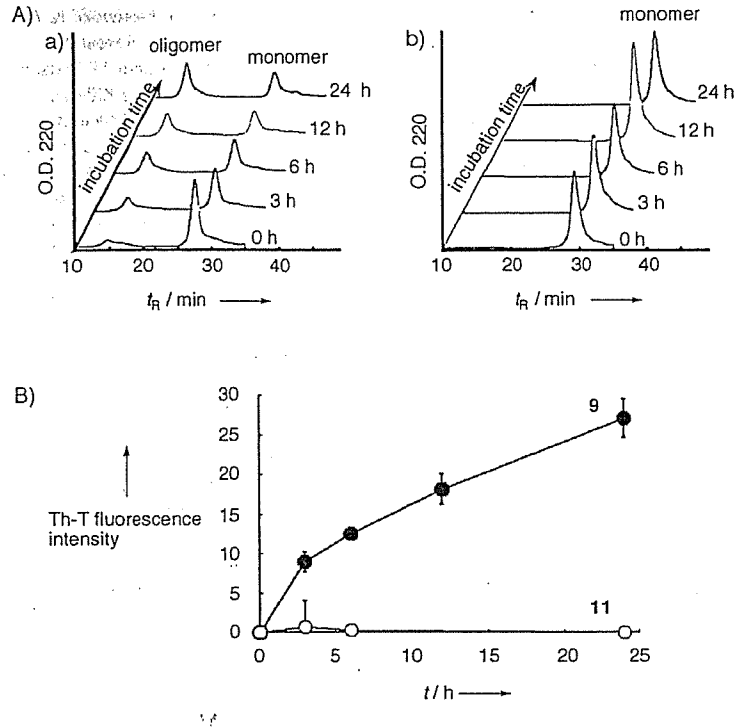

Figure 4. Peptide aggregation determined A) by size-exclusion chromatography—a) Aβ1–42 (**9**); b) click peptide **11**—and B) by Th-T assay.

37 °C; this suggests that click peptide **11** is sufficiently stable for use in biological assays under ambient light.[34] Peptide **11** was stable for at least 24 h in buffer (pH 7.4) solution at −20 °C and for three months either in the solid state or in DMSO solution at −20 °C. On the other hand, when a solution of **11** in PBS (pH 7.4, 20 μM peptide, 1 mM DTT) was photoirradiated with UV pulses for 15 min, the Nvoc group-derived absorption band at around 355 nm completely disappeared, indicating that the Nvoc group in **11** had been quantitatively removed by photolysis. We also confirmed that Aβ1–42 (**9**) was quantitatively recovered from **11** after photolysis followed by incubation at 37 °C for 30 min to induce migration. No by-products arising from 4,5-dimethoxy-2-nitrosobenzaldehyde co-released with **9** by photolysis were observed.

These results suggest that 1) click peptide **11** did not exhibit any selfassembling nature under physiological conditions, 2) photoirradiation of **11** and subsequent *O–N* intramolecular acyl migration rapidly afforded intact **9** in situ, while **11** was stable under nonphotolytic or storage conditions, and 3) no additional fibril-inhibitory auxiliaries were required. This method provides a novel system useful for investigation of the biological dynamism of Aβ1–42 in AD by inducible activation of Aβ1–42 self-assembly. Additionally, a fundamental drawback of the caged strategy for large peptides or proteins is that a small photocleavable group is not always able to mask their biological activities. This drawback would be overcome by our "click peptide" strategy in which the inherent properties can be masked by simple isomerization of the backbone structure from *N*-acyl peptide to *O*-acyl isopeptide at hydroxyamino acid residues such as Ser and Thr. This method should open doors for the development of novel and useful phototriggerable tools in chemical biology and medical science.

5) Conclusion

In 2003, when we conceived the idea that the synthesis of more hydrophilic "*O*-acyl isopeptides" derived from peptides containing difficult sequences might overcome the solubility problem in HPLC purification, we made the surprising discovery that the "*O*-acyl isopeptide" could improve not only the solubility in various media, but also coupling and deprotection efficacy during solid-phase peptide synthesis through the modification of the nature of the difficult sequence. The isomerization of the peptide backbone at only one position in the whole peptide sequence—that is, the formation of one single ester bond—could significantly change the unfavorable secondary structure of the peptide. This finding led to the discovery of the "*O*-acyl isopeptide method" as a novel synthetic method in the field of peptide chemistry whose efficacy has recently been confirmed by Mutter et al.[21a,c] and by Carpino et al.[21b] In research oriented towards chemical biology we have applied this method to the synthesis of a novel "click peptide" precursor for Aβ1–42. This click peptide did not exhibit any self-assembling nature under physiological conditions, because of the presence of a single ester moiety, but was able to undergo a migration to form the original Aβ1–42 in a quick and one-way conversion reaction (so-called "click"). Because the difficulties involved in handing Aβ1–42 in synthesis and in biological experiments, are an impediment in progress in Aβ1–42-related Alzheimer's disease research, we expect that the "click peptide" method should contribute to clarifying the currently unexplained processes of Alzheimer's disease. Moreover, it has recently been ascertained that the pathological self-assembly of inherent peptides or proteins and their subsequent aggregation into amyloidogenic deposits is associated with many diseases,[38] such as prion protein in prion disease, α-synuclein in Parkinson's disease, and islet amyloid polypeptide in type 2 diabetes, as well as Aβ1–42 in AD, so we hope that the "click peptide" strategy may be widely applied to these amyloid-related peptides or proteins.

Acknowledgements

This research was supported in part by the "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of the Japanese Government, and the 21st Century COE Program from MEXT. Y.S. is grateful for Research Fellowships of JSPS for Young Scientists. We thank Dr. J.-T. Nguyen for his help in preparing the manuscript.

Keywords: Alzheimer's disease · amyloid peptide · click peptide · O-acyl isopeptide method · self-assembly

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Received: March 22, 2006
Published online on August 17, 2006

Journal of Peptide Science
J. Pept. Sci. 2006; **12**: 823–828
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.817

'Click peptide': a novel '*O*-acyl isopeptide method' for peptide synthesis and chemical biology-oriented synthesis of amyloid β peptide analogues

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Received 28 July 2006; Revised 20 September 2006; Accepted 20 September 2006

Abstract: After over a decade of studies on aspartic protease inhibitors and water-soluble prodrugs, we have been developing a novel method, since 2003, called '*O*-acyl isopeptide method', for the synthesis of peptides containing difficult sequences. With our recent discoveries of '*O*-acyl isodipeptide unit' and the 'racemization-free segment condensation method', this method has further evolved as a general synthetic method for peptides. Moreover, 'Click Peptide', which could be a powerful tool for identifying the pathological functions of amyloid β peptides in Alzheimer's disease, represents a valuable use of the isopeptide method in Chemical Biology-oriented research. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *O*-acyl isopeptide method; Alzheimer's disease; amyloid β peptide; click peptide; difficult sequence

INTRODUCTION

Since 2003, we have been developing a novel method, called '*O*-acyl isopeptide method', for the synthesis of peptides containing difficult sequences in which a native amide bond at a hydroxyamino acid residue, such as Ser being isomerized to an ester bond, is followed by an *O*–*N* intramolecular acyl migration reaction [1–10]. Recently, the '*O*-acyl isopeptide method' began to be widely utilized by several other groups [11–15]. In chemical biology-oriented research, we developed a novel 'Click Peptide' based on the *O*-acyl isopeptide method to study the inherent biological functions of native peptides or proteins (Figure 1) [3–7,9].

O-ACYL ISOPEPTIDE METHOD

Several years ago, when we tried to synthesize some peptide derivatives, including phenylnorstatine, for the study of aspartic protease inhibitors [16–18], some of the synthesized compounds could not be purified in preparative scale HPLC owing to their extremely low solubility in various solvents (Figure 2(A)). Thus, these peptide derivatives were considered to be the so-called 'difficult sequence'-containing peptides [19,20].

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On the other hand, for over a decade, we studied the pH-dependent '*O*–*N* intramolecular acyl migration [21,22]'-type water-soluble prodrugs of the peptide mimetic HIV-1 protease inhibitors [23,24]. These prodrugs, which are *O*-acyl isoforms of parent drugs possessing α-hydroxy-β-amino acids, had higher water solubility because of a newly formed and ionized amino group. Moreover, migration to the *N*-acyl parent drugs could be transacted with no side reaction under physiological conditions.

As a consequence, in 2003, we considered that the hydrophilic '*O*-acyl isopeptides' derived from the phenylnorstatine-containing peptide derivatives would overcome the solubility problem in HPLC purification (Figure 2(B)). However, we had a surprising discovery in this research, which showed that not only did the '*O*-acyl isopeptide' possess a higher solubility in various media, but also that the coupling and deprotection efficacy during solid-phase peptide synthesis (SPPS) was improved by modifying the nature of the difficult sequence [1,2]; namely, the isomerization of the peptide backbone from the *N*-acyl to *O*-acyl isopeptide structure, i.e. formation of one single ester bond, significantly changed the unfavorable secondary structure of the native peptides. Thus, this finding led to the development of the '*O*-acyl isopeptide method' as a novel synthetic method in the field of Peptide Chemistry.

We also designed an '*O*-acyl isodipeptide unit', e.g. Boc–Ser/Thr(Fmoc–Xaa)–OH. The use of *O*-acyl isodipeptide units, in which the racemization-inducing

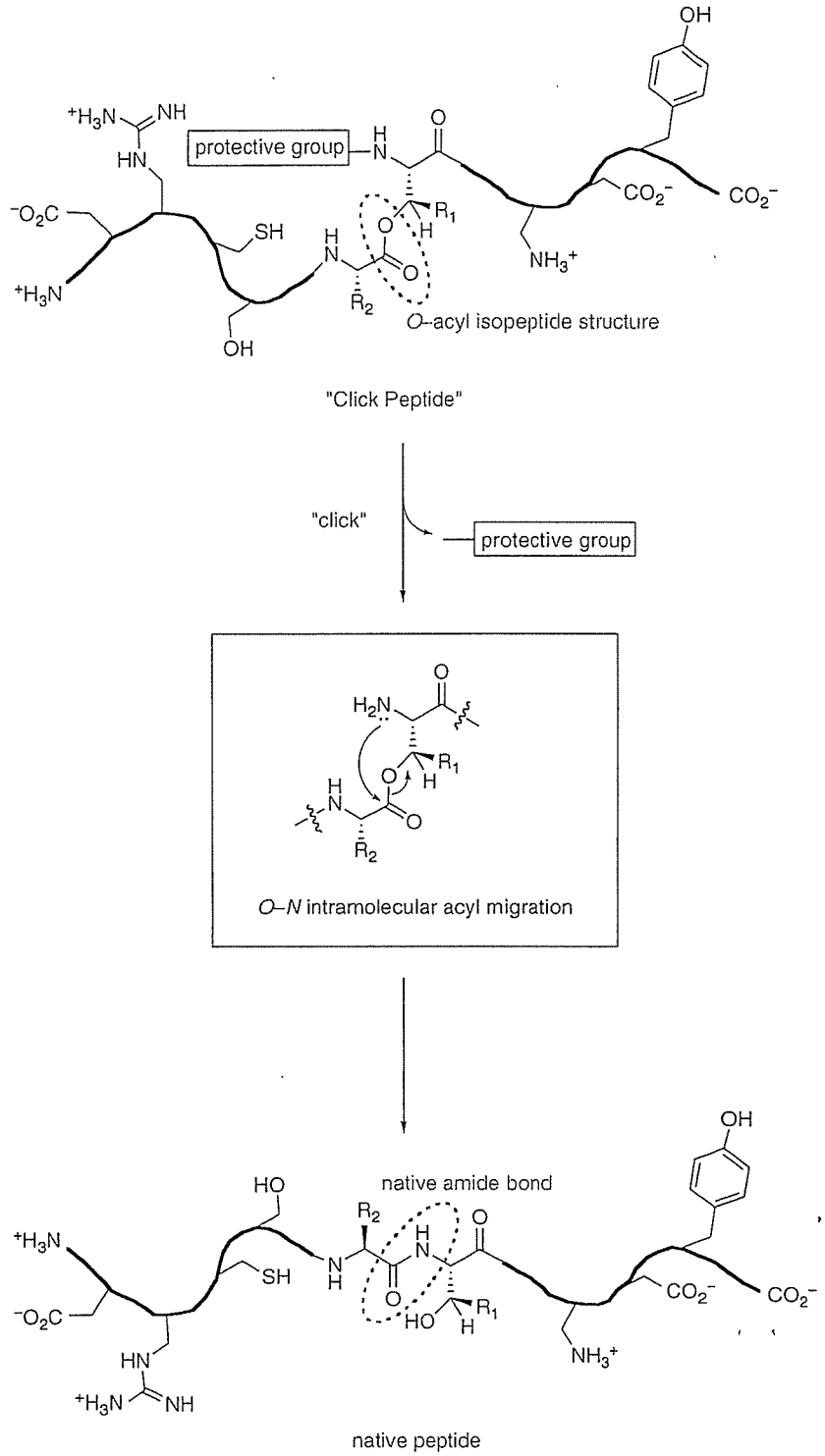

Figure 1 'Click Peptide' based on the '*O*-acyl isopeptide method'.

esterification reaction on the resin could be omitted, allows the application of the '*O*-acyl isopeptide method' to fully automated protocols for the synthesis of long peptides or proteins (Figure 3) [8]. Additionally, very recently, we developed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' [10].

The Application of *O*-Acyl Isopeptide Method

Moreover, we have successfully applied the '*O*-acyl isopeptide method' to the chemical biology-oriented synthesis of the Alzheimer's disease (AD)-related amyloid β peptide (Aβ) 1–42 analogues, leading to the development of pH- or photo-triggered 'Click Peptide'

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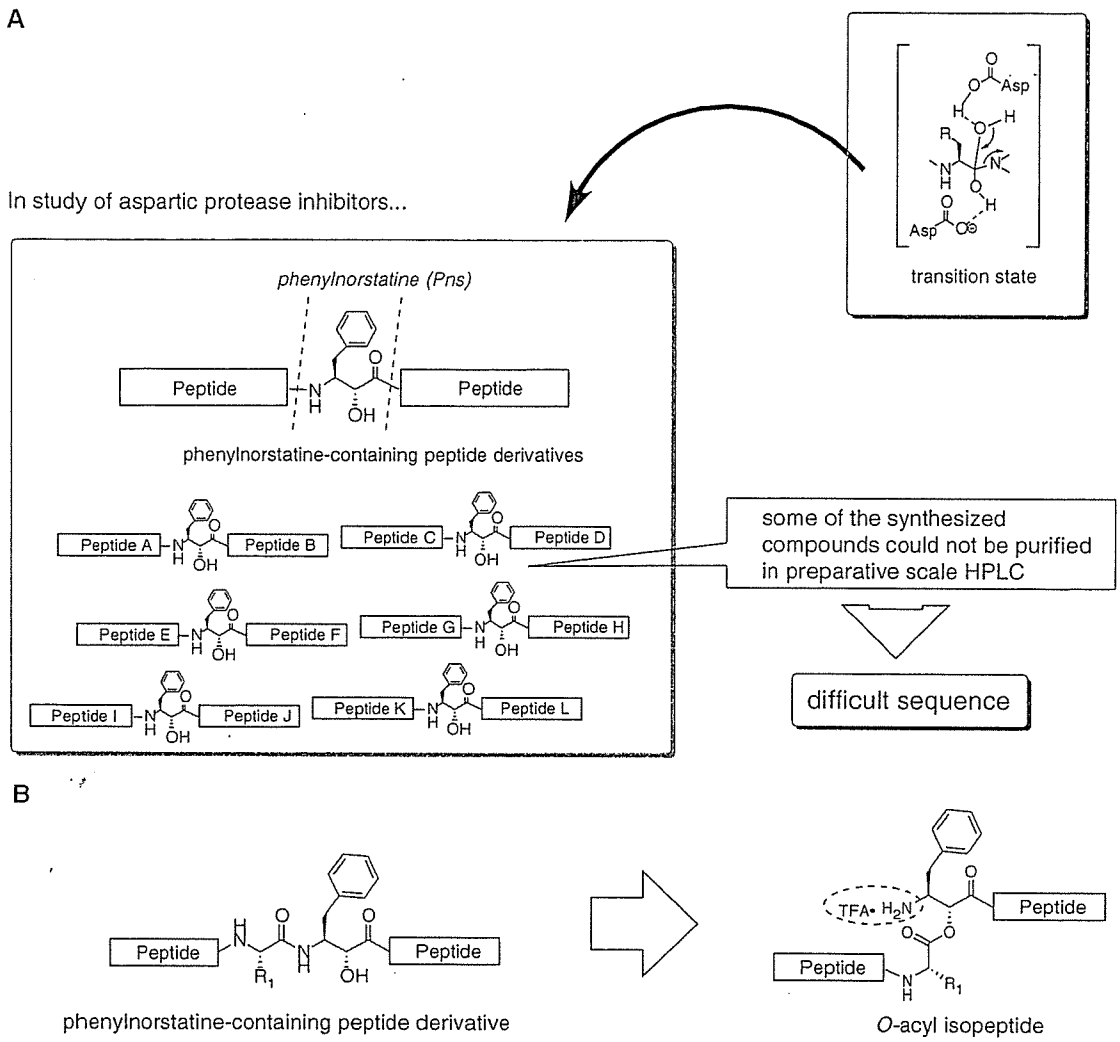

Figure 2 (A) 'Difficult sequence' in our studies of aspartic protease inhibitors, (B) design of *O*-acyl isopeptide derived from phenylnorstatine-containing peptide derivative.

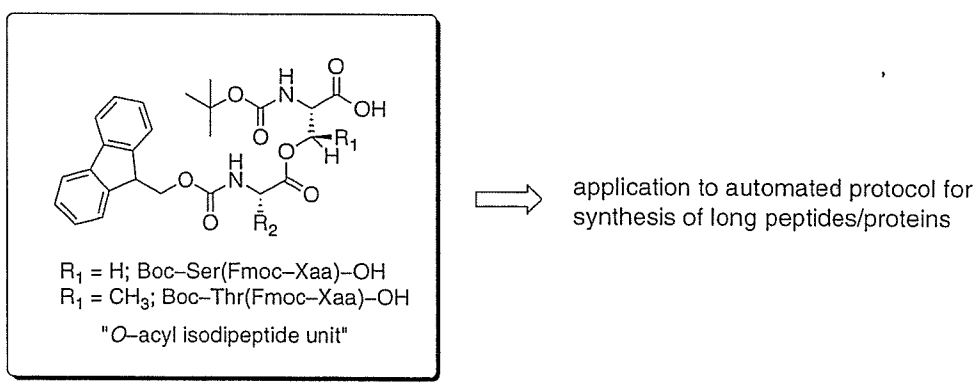

Figure 3 General structure of *O*-acyl isodipeptide unit: application of the '*O*-acyl isopeptide method' to fully automated protocol.

(Figure 1) [3–7,9]. The 'Click-Peptide' did not exhibit the self-assembling nature under physiological conditions because of one single ester, and could migrate to the original Aβ1–42 with a quick and easy one-way conversion reaction (so-called 'click') via the *O–N* intramolecular acyl migration. A clear understanding of the pathological mechanism of Aβ1–42, a currently unexplained process, would be of great significance in the discovery of novel drug targets against AD [25–28]. Currently, the difficulties in handling Aβ1–42, because

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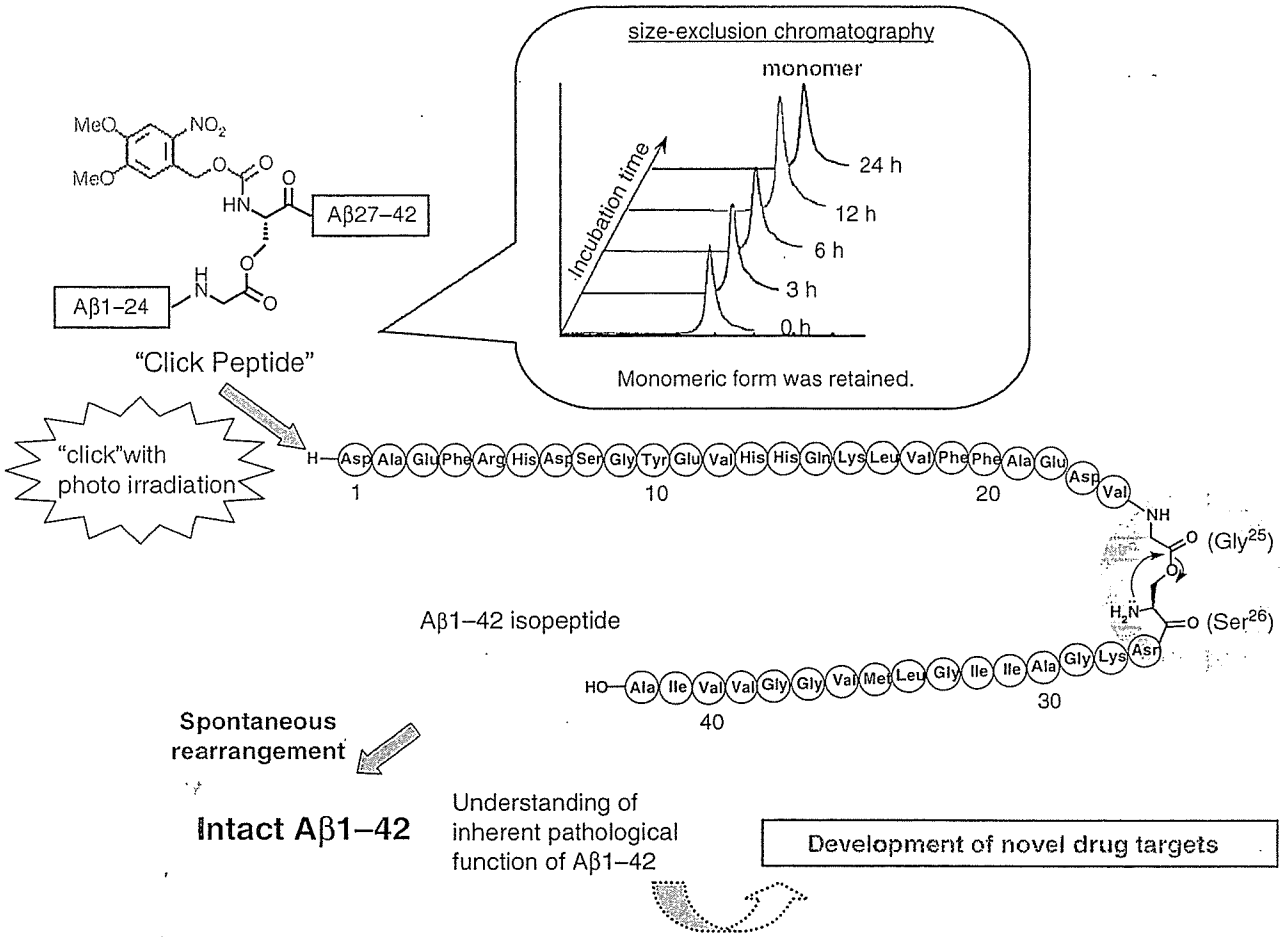

Figure 4 Photo-triggered click peptide. The production of Aβ1–42 by photo-triggered click followed by *O–N* intramolecular acyl migration reaction of Aβ1–42 isopeptide.

of its highly aggregative nature, hamper the progress of Aβ1–42-related AD research [29–32]. The 'Click Peptide' method would open the doors for investigation of the biological functions of Aβ1–42 in AD by inducible activation of Aβ1–42 self-assembly (Figure 4).

Interestingly, shortly after we disclosed the '*O*-acyl isopeptide method' [1,2], several other groups also confirmed the efficacy of this method. Carpino *et al.* synthesized the Jung–Redemann 26-residue peptide efficiently by utilizing the '*O*-acyl isopeptide method', whereas this peptide could not be synthesized by standard SPPS [12]. By carefully evaluating the appropriate protecting group, stability of the ester bond during assembly, and occurrence of side reactions, they concluded that the efficacy of the '*O*-acyl isopeptide method' was comparable to that of the pseudoproline method [15]. Börner *et al.* also synthesized the *O*-acyl isopeptide for efficient preparation of poly(ethylene oxide)–peptide conjugates [14]. Moreover, Mutter *et al.* confirmed by circular dichroism (CD)-based analyses that the secondary structure of *O*-acyl isopeptide structure is significantly different from that of the corresponding *N*-acyl native peptides [11,13], which agrees with our hypothesis. These reports indicate that the '*O*-acyl isopeptide method' is widely advantageous for peptide preparation by disrupting the unfavorable secondary structures of the native peptides.

CONCLUSION

Classical *O–N* intramolecular acyl migration was revived by our group as a powerful key reaction in the field of modern medicinal chemistry in the development of water-soluble prodrugs. After more than a decade of prodrug studies, we recently disclosed the '*O*-acyl isopeptide method' as a novel synthetic method in the field of peptide chemistry and its application to chemical biology-oriented synthesis of Aβ analogues, leading to the development of 'Click Peptide' (Figure 5). We hope that the strategy using the '*O*-acyl isopeptide method', in which a simple isomerization to an *O*-acyl isopeptide remarkably and temporarily changes the physicochemical properties of the native peptide and an *O–N* intramolecular acyl migration triggers the native amide bond formation under physiological conditions,

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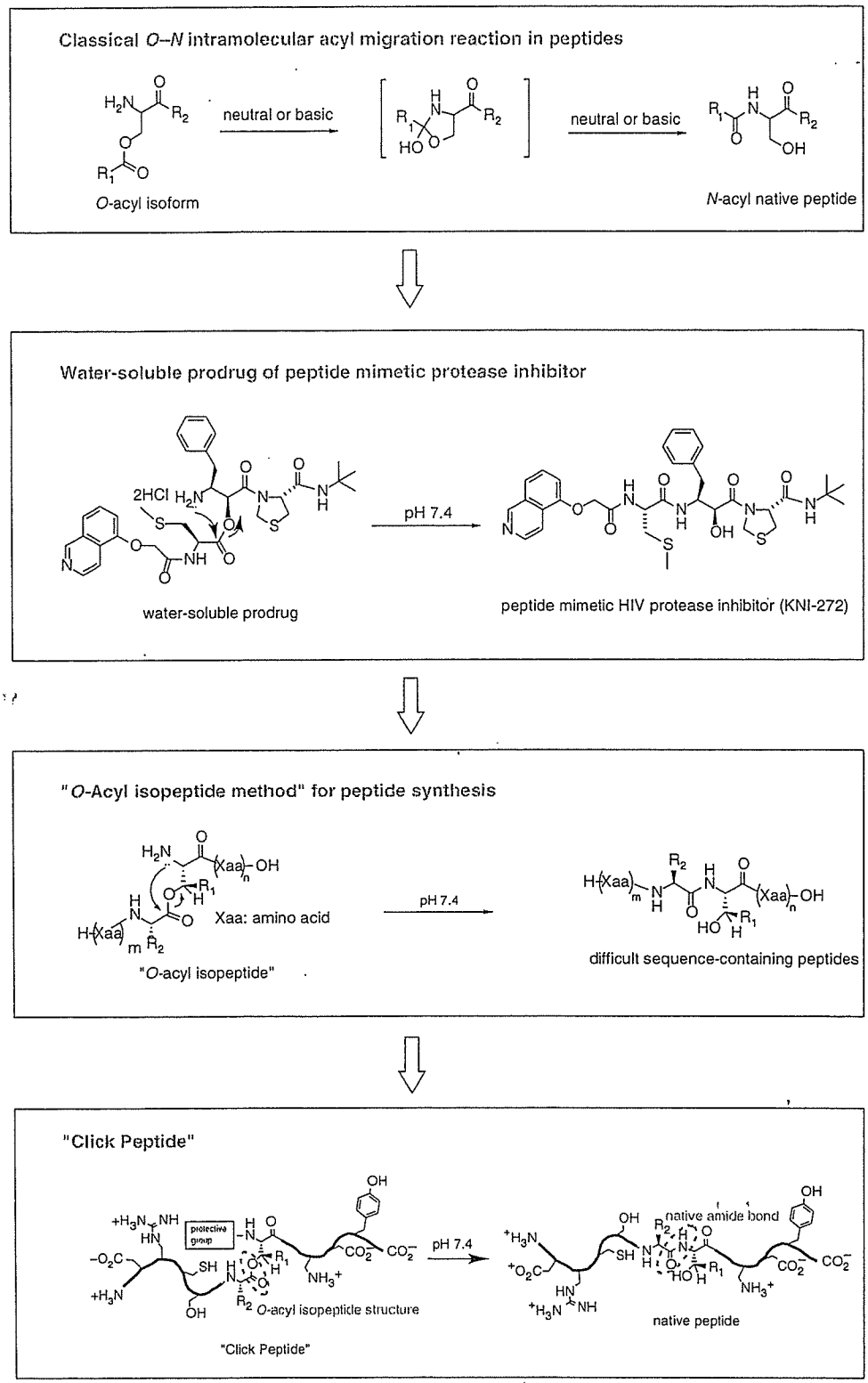

Figure 5 Our workflow on peptide science based on the '*O*–*N* intramolecular acyl migration reaction'.

will further contribute to the study of peptides and proteins. Examples of such studies include the studies of membrane peptides/proteins that are difficult to handle in various conditions because of their high self-assembling characters.

Acknowledgements

This research was supported in part by the 'Academic Frontier' Project for Private Universities, a matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of the

Japanese Government, and the 21st Century COE Program from MEXT. Y.S., M.S., and T.O. are grateful for Research Fellowships of JSPS for Young Scientists. We thank Dr J-T. Nguyen for his help in preparing the manuscript.

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Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells

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Contributed by Malcolm A. Martin, September 20, 2006

The narrow host range of human immunodeficiency virus type 1 (HIV-1) is caused in part by innate cellular factors such as apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) and TRIM5α, which restrict virus replication in monkey cells. Variant HIV-1 molecular clones containing both a 21-nucleotide simian immunodeficiency virus (SIV) Gag CA element, corresponding to the HIV-1 cyclophilin A-binding site, and the entire SIV *vif* gene were constructed. Long-term passage in a cynomolgus monkey lymphoid cell line resulted in the acquisition of two nonsynonymous changes in *env*, which conferred improved replication properties. A proviral molecular clone, derived from infected cells and designated NL-DT5R, was used to generate virus stocks capable of establishing spreading infections in the cynomolgus monkey T cell line and CD8-depleted peripheral blood mononuclear cells from five of five pig-tailed macaques and one of three rhesus monkeys. NL-DT5R, which genetically is >93% HIV-1, provides the opportunity, not possible with currently available SIV/HIV chimeric viruses, to analyze the function of multiple HIV-1 genes in a broad range of nonhuman primate species.

APOBEC3 | host range | monkey model | TRIM5α | cyclophilin A

The narrow host range of human immunodeficiency virus type 1 (HIV-1) has been a major impediment for developing tractable animal models for studies of viral pathogenesis and vaccine development. Because simian immunodeficiency virus (SIV) has a genomic organization similar to that of HIV-1 and some SIV strains cause disease in Asian macaques, SIV/HIV chimeric viruses (SHIVs) were generated to assess the role of some HIV-1-encoded proteins in nonhuman primates (1–3). The commonly used SHIVs contain the HIV-1 *tat*, *rev*, *vpu*, and *env* genes inserted into an SIVmac239 genetic backbone; efforts to extend the incorporated HIV-1 gene segment to include *pol* and *gag* sequences have resulted in viruses unable to replicate in monkey cells (ref. 1; unpublished data). Although SHIVs have proven useful in characterizing the immune responses to primate lentiviruses (4, 5), and specifically, the role of antibodies directed against the HIV-1 envelope glycoprotein (6, 7), the absence of the other HIV-1 structural proteins has restricted analyses of their function *in vivo*.

It is now appreciated that many mammalian species encode factors conferring resistance to retroviral infections. Some, such as the apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3) family of cytidine deaminases, modify minus strand viral DNA during reverse transcription, resulting in either its degradation or its integration into host chromosomal DNA as a hypermutated provirus (8–10). The retroviral inhibitory effect of APOBEC3G results from its packaging into progeny virions during particle assembly (11–12). The deleterious activities of APOBEC3G are countered by lentiviral Vif proteins, which prevent the encapsidation of APOBEC3G into nascent virions (13–16). The sensitivity of APOBEC3G from different animal species to the Vif proteins expressed by different viruses varies widely. For example, although HIV-1 Vif can potently suppress human APOBEC3G, it is not effective against rhesus monkey (RhM) APOBEC3G, explaining in part the restriction of HIV-1 replication in macaque cells (11).

Another recently described restriction factor, TRIM5α, targets incoming viral capsids, and it blocks retroviral replication in a species-specific manner (17–19). For example, TRIM5α from RhMs potently suppresses HIV-1 but not SIV infectivity in monkey cells (19). Although its mechanism of action is still unclear, TRIM5α restriction is thought to affect virus uncoating, thereby blocking subsequent steps in the replication cycle (20). Cyclophilin A (CypA), which binds to a proline-rich loop on the surface of the HIV-1 capsid (CA) protein, augments HIV-1 infection in human cells and inhibits its replication in monkey cells (21, 22). Recent reports suggest that by binding to HIV-1, CypA may modulate the conformation of the virion core, rendering it sensitive to TRIM5α restriction in simian cells (23, 24).

In this work, we have generated HIV-1 derivatives, which carry only the SIVmac239 *vif* gene and a short 7-aa segment from SIV *gag* corresponding to the HIV-1 CypA-binding loop. Molecularly cloned viruses bearing these two SIV regions are able to establish spreading infections in a cynomolgus monkey (CyM) T cell line and CD8-depleted PBMCs from pig-tailed macaques (PtMs) and RhMs. These results indicate that the incorporation of two SIV gene segments into the HIV-1 genome can effectively counter two known species-specific restriction factors that block virus replication in monkey cells. They raise the possibility of generating HIV-1 derivatives, containing all of its structural proteins and capable of infecting macaque monkeys.

Results

Construction and Characterization of HIV-1 Molecular Clones Containing CA and Vif Sequences from SIVmac239. Three proviral DNA constructs were generated to counteract the restriction of HIV-1 replication in macaque monkey cells. In the first, the entire 214-aa Vif ORF from SIVmac239 was amplified by PCR and

Author contributions: K.K. and A.A. designed research; K.K., T.I., M.A.M., B.K., K.H., T.Y., M.F., T.U., and A.A. performed research; K.K. and A.A. analyzed data; and K.K., M.A.M., and A.A. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: AGM, African green monkey; APOBEC3G, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G; CA, capsid; CyM, cynomolgus monkey; CypA, cyclophilin A; PBMC, peripheral blood mononuclear cell; p.i., postinfection; PtM, pig-tailed monkey; RhM, rhesus monkey; RT, reverse transcriptase; SHIV, SIV/HIV chimeric virus; SIV, simian immunodeficiency virus; SIVmac, simian immunodeficiency virus isolated from rhesus macaques; VSV-G, vesicular stomatitis virus type G.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB266485, AB266486, AB226487, and AB266488 for pNL-DT5R and APOBEC3Gs from AGM, human, and CyM, respectively).

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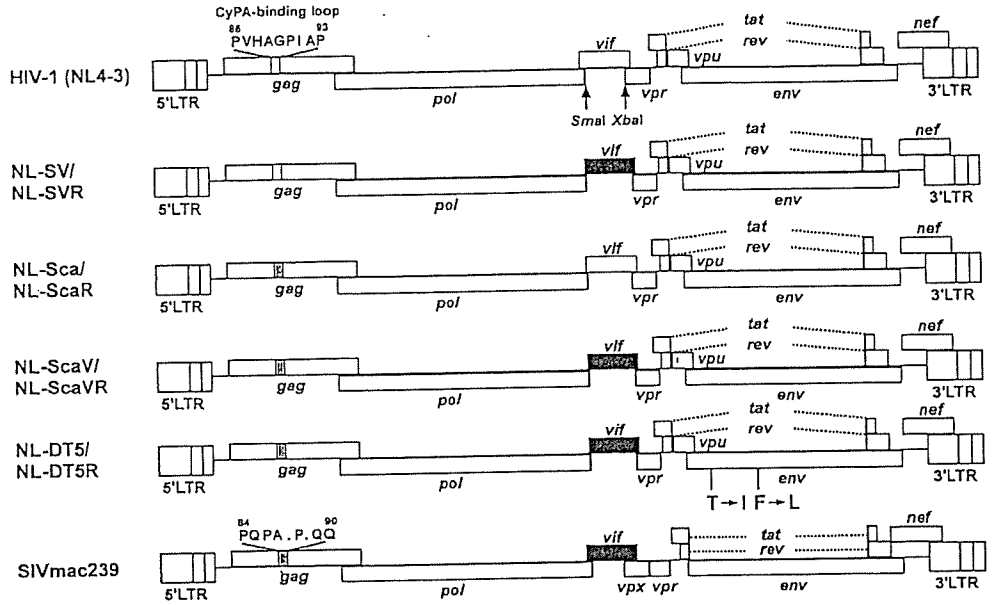

Fig. 1. Structure of chimeric clones between HIV-1 NL4-3 and SIVmac239 in this study. Eight chimeric proviral clones shown here were generated from a pNL4-3 derived vector pNL-SX (25) as described in *Materials and Methods*. Each chimeric clone has the entire *vif* (black area) and/or partial *gag* (gray area; analog of HIV-1 CypA-binding loop) of SIVmac239 as shown. For insertion of *vif* into the clones, the SmaI–XbaI site in pNL-SX was used. The two amino acid changes in *env* unique to pNL-DT5 and pNL-DT5R are indicated.

inserted into SmaI–XbaI-digested pNL-SX, a pNL4-3-derived vector, previously used for functional analyses of HIV-1 *vif* genes (25). This SIV Vif-encoding construct was designated NL-SV (Fig. 1). Because of the reported association of CypA with HIV-1 sensitivity to TRIM5α during infections of cells from Old World monkeys (21, 22), the 9-aa CypA-binding loop in NL4-3 was converted to the 7-residue SIVmac239 CA analog by site-directed mutagenesis of the pNL-SX vector carrying the HIV-1 *vif* gene (26). This construct was designated NL-Sca (Fig. 1). A final clone, containing both SIV elements, was generated by inserting SIV *vif* into NL-Sca and designated NL-ScaV (Fig. 1).

Expression of the lentiviral genes present in the three newly derived cloned proviruses was assessed by immunoblot analyses of lysates prepared from transfected 293T cells. The production of Gag, Pol, Env, Vpu, and Nef proteins directed by all three constructs was comparable with that observed with the parental pNL4-3; levels of Vpr expression, however, were markedly reduced (data not shown). The latter was subsequently shown to be caused by the presence of the TCT trinucleotide, introduced into the pNL-SX vector to generate the XbaI cloning site (25). When the TCT was removed by site-specific mutagenesis, Vpr expression was restored to wild-type levels in cells transfected by all three constructs (data not shown). The "Xba site-repaired" clones were designated NL-SVR, NL-ScaR, and NL-ScaVR, respectively, as indicated in Fig. 1.

HIV-1 Constructs Bearing the SIV *vif* Gene Are Able to Suppress the Inhibitory Effects of Simian APOBEC3Gs. It has been previously reported that RhM and African green monkey (AGM) APOBEC3Gs are resistant to HIV-1 *vif*, possibly explaining, in part, the restriction of HIV-1 replication in cells from Old World monkeys (11). To determine whether the simian APOBEC3Gs could block HIV-1 constructs carrying the SIVmac239 *vif* gene, VSV-G-pseudotyped viruses were generated in 293T cells in the presence of different APOBEC3Gs. For this experiment, species-specific APOBEC3G cDNAs were prepared from H9 (human), HSC-F (CyM) (27), and Vero (AGM) cells by RT-PCR and inserted into pcDNA3.1-FLAG, an expression vector containing an epitope tag, as described in *Materials and Methods*. Comparable levels of human, CyM, and AGM APOBEC3Gs were produced in transfected 293T cells, as monitored by immunoblotting by using anti-FLAG antibodies (Fig. 2*A*). The progeny virions generated in cells expressing human or the two monkey APOBEC3Gs were collected from culture supernatants at 48 h, and their infectivities were assayed in MAGI cells (Fig. 2*B*). Not unexpectedly, the replication of viruses (NL4-3 and NL-ScaR) bearing the HIV-1 *vif* gene and produced in cells expressing CyM and AGM APOBEC3Gs was potently suppressed. In contrast, the constructs (NL-SVR and NL-ScaVR) carrying the SIVmac239 *vif* gene were refractory to the effects of both CyM and AGM APOBEC3Gs. When the expression of the SIVmac *vif* gene in NL-ScaVR was abrogated by a frameshift mutation, the resulting virus (NL-ScaVR-dBgl) became sensitive to all three APOBEC3Gs, and its infectivity was markedly reduced. These results indicate that under the same experimental conditions in which simian APOBEC3Gs restrict wild-type HIV-1, the derivative clones expressing SIV Vif direct the production of virions able to replicate in MAGI cells.

HIV-1 Constructs Carrying a 7-Amino Acid SIV CA Element Exhibit Increased Replication in Simian Cells. The capacity of HIV proviruses bearing the SIV Gag analog of the HIV-1 CypA-binding loop to escape restriction in simian cells was assessed by using VSV-G-pseudotyped viruses (NL4-3, NL-ScaR, NL-SVR, and NL-ScaVR) in single-cycle replication assays by using human (293T), RhM (LLC-MKII), and owl monkey (OMK637) cell lines (Fig. 2*C*). Three-fold serial dilutions of each virus stock were added to the cultures, and the amounts of p24 Gag protein present in cell lysates 72 h postinfection (p.i.) was determined by ELISA. All four viruses expressed p24 with similar efficiencies in human cells. In contrast, the NL-ScaR and NL-ScaVR derivatives, both of which carry the SIV CA element, produced substantially more p24 Gag in monkey cells than the constructs (NL4-3 and NL-SVR) bearing the HIV-1 CypA-binding loop. This effect was particularly striking in owl monkey cells in which constructs carrying the SIV CA expressed 50-fold more viral protein. Not unexpectedly, the presence of SIV *vif* alone in NL-SVR did not result in increased p24 Gag production com-

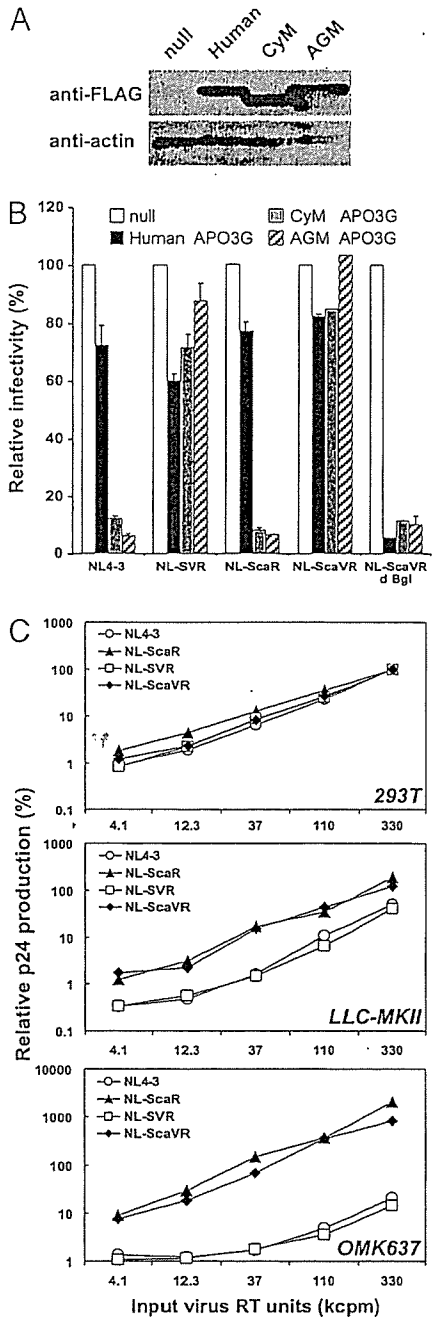

Fig. 2. Single-cycle replication properties of HIV-1 chimeric clones. (*A*) The expression of human, CyM, and AGM APOBEC3Gs containing the FLAG epitope was monitored in 293T cells by immunoblotting 48 h posttransfection. (*B*) VSV-G-pseudotyped viruses were prepared in 293T cells cotransfected with one of the three expression vectors for APOBEC3G (human, CyM, and AGM), and their infectivity was examined in MAGI cells. Infectivity relative to that of virus produced in the absence of APOBEC3G (null) is indicated. (*C*) Production of p24 Gag in human (293T), RhM (LLC-MKII), and owl monkey (OMK637) cells after infection with increasing amounts of the indicated VSV-G-pseudotyped viruses was measured on day 3 p.i. Expression levels of p24, relative to that generated by the virus sample with the highest RT activity in 293T cells, are indicated.

pared with wild-type HIV-1 in this single-cycle replication assay. The results shown in Fig. 2*C* indicate that the incorporation of a 21-nucleotide SIV *gag* gene element into HIV-1 proviral DNA is sufficient to suppress the endogenous restriction factors resident in RhM and owl monkey cells.

An HIV-1 Derivative Containing both SIV *vif* and the SIV CA Element Is Able to Establish Spreading Infections in a Monkey Lymphocyte Cell Line. Although single-cycle replication experiments using pseudotyped retroviral particles like those shown in Fig. 2 *B* and *C* can furnish valuable information about virus entry, uncoating, reverse transcription, integration, and the production of viral proteins, they provide no data about the functional properties of the progeny virions that are generated. The latter information accrues from spreading multicycle infections. Toward this end, NL-ScaR, NL-SVR, and NL-ScaVR virus stocks, prepared from transfected 293T cells and normalized for equivalent amounts of reverse transcriptase (RT) activity, were used to infect human (M8166) and cynomolgus (HSC-F) cells. HSC-F is a $CD4^+CXCR4^+CCR5^-$ CyM T cell line originally immortalized by *Herpesvirus saimiri* (27). Both cell types were also infected with similar amounts of the parental NL4-3 and SIVmac239, which served as controls. As shown in Fig. 3*A*, all of the viruses did, in fact, establish spreading infections in M8166 cells, although the three bearing the SIV *vif* gene (SIVmac239, NL-SVR, and NL-ScaVR) reached lower levels of peak RT activity compared with NL4-3 and NL-ScaR.

A completely different result was obtained during infections of the CyM cell line. As expected, SIVmac239 readily established a spreading infection, which peaked on day 6 p.i.; wild-type NL4-3 produced no measurable progeny virions (Fig. 3*B*). Of the three NL4-3 derivatives carrying SIV sequences, only NL-ScaVR exhibited any infectivity, which became detectable on day 15 p.i. The delayed appearance of NL-ScaVR progeny is reminiscent of previously described second-site revertants of HIV-1 mutants, which acquire changes during extended tissue culture passage that confer augmented replicative properties (28, 29). Therefore, to characterize more fully the late emerging virus, new and independent infections of HSC-F cells were initiated by using both NL-ScaV- and NL-ScaVR-derived virus preparations as inocula; in both cases, the production of progeny virions was again markedly delayed (data not shown). Culture supernatants from the NL-ScaV infection were collected on days 39 and 51 p.i., normalized for RT activity, and used as inocula for infections of fresh HSC-F cells. As shown in Fig. 3*C*, the viruses harvested on days 39 and 51 both exhibited accelerated replication kinetics compared with the original NL-ScaV virus, suggesting that long-term passage in HSC-F had resulted in the acquisition of genetic alterations.

Molecular Cloning and Characterization of an HIV-1 Derivative Able to Cause Spreading Infections in Macaque Primary Cells. The emergence of virus exhibiting an augmented replication phenotype prompted us to initiate the molecular cloning of cell-associated viral DNA collected from HSC-F cultures infected with the "sup 51" inoculum on day 18 p.i. Integrated proviruses were amplified from genomic DNA as two overlapping fragments by PCR, and virus stocks were prepared from 293T cells after transfection with reconstructed full-length clones. The replication properties of one of the infectious clones obtained (NL-DT5) is shown in Fig. 3*D*. Although production of progeny virus was delayed compared with that directed by SIVmac239, NL-DT5 still exhibited robust infection kinetics and released more particle-associated RT activity than the SIV control.

Sequencing of NL-DT5 DNA revealed that it had acquired four nucleotide changes, compared with NL-ScaV, during the 51-day passage in HSC-F cells. Two were nonsynonymous changes in *env* (nts 6633 and 7043), resulting in T110I (V1) and F247L (C2) substitutions in gp120. One of the other two was a synonymous change in the Pro coding sequence (nt 2300) and the other was a G to A substitution in the U3 region of the 3′ LTR. The functional significance of these changes is not presently known. Because NL-DT5 was derived from cells originally infected with NL-ScaV, the XbaI cloning site present upstream

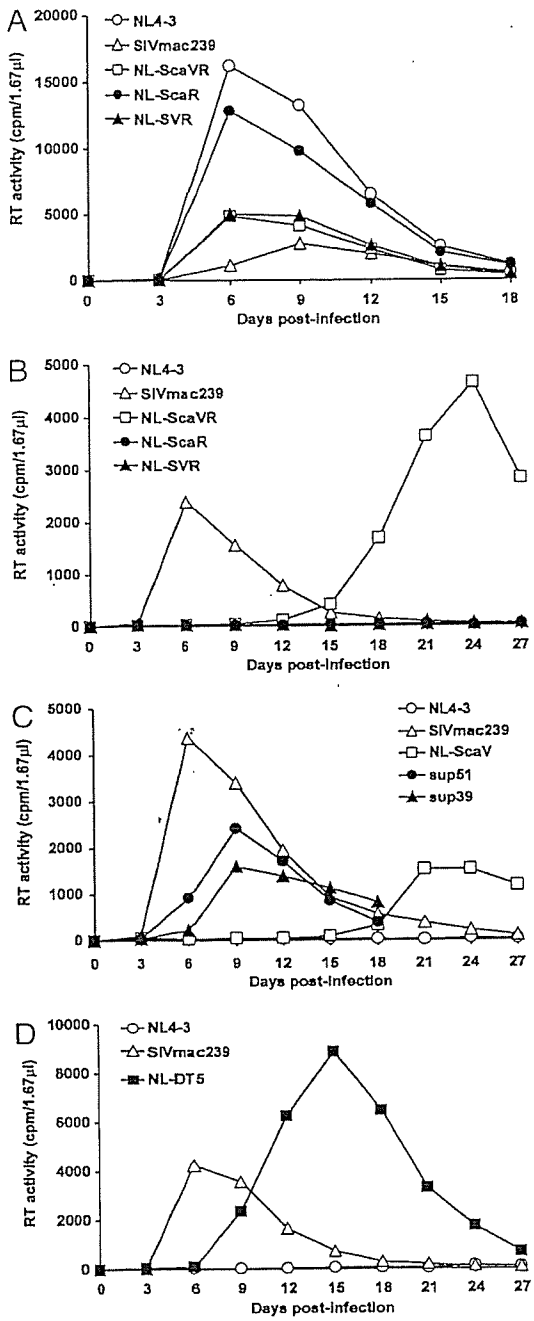

Fig. 3. Multicycle growth potential of various chimeric viruses in human and monkey lymphocyte cell lines. Virus samples were prepared from 293T cells transfected with the indicated proviral clones, and they were inoculated into human M8166 (*A*) or CyM HSC-F (*B*) cells. HIV-1 NL4-3 and SIVmac239 served as controls. (*C* and *D*) Growth properties of viruses generated in infected HSC-F cells. Culture supernatants from NL-ScaV-infected HSC-F cells collected on days 39 and 51 p.i. (sup39 and sup51 in *C*) and from 293T cells transfected with a molecular clone derived from sup51 (NL-DT5 in *D*) were inoculated into HSC-F cells. NL4-3, SIVmac239, and NL-ScaV from transfected 293T cells served as controls. Virus replication was monitored by RT activity released into the culture supernatants.

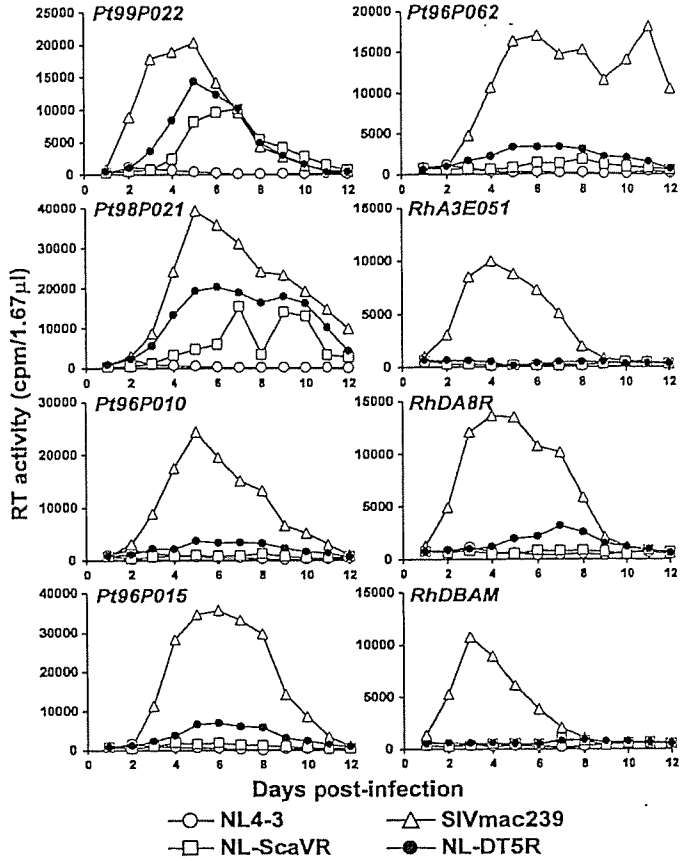

Fig. 4. Growth potential of the chimeric viruses in CD8-depleted PBMCs from PtM and RhM. Virus samples were prepared from 293T cells transfected with the proviral clones indicated at the bottom, and they were infected into CD8-depleted PBMCs by spinoculation (30). Virus replication was monitored daily by RT production in the culture supernatants. HIV-1 NL4-3 and SIVmac239 served as controls. Animal identifications are indicated at the top of each panel.

from *vpr* was repaired by deleting the TCT trinucleotide, as described earlier, and the resulting molecular clone was designated NL-DT5R.

Because the ultimate use of NL-DT5R would be as a virus inoculum in nonhuman primate studies, a more rigorous test of its infectivity would be replication in macaque PBMCs. In an initial experiment, unfractionated and ConA-activated PBMC from five PtM and three Indian origin RhM were infected with NL-DT5R, NL4-3, and SIVmac239 by spinoculation (30). Production of SIVmac239 progeny virions was initially detected on day 3 and peaked on day 6 p.i.; no replication of NL4-3 or NL-DT5R was observed during the 12-day course of this infection (data not shown). In contrast to these results, NL-DT5R was able to establish spreading infections in five of five PtM and one of three RhM PBMC preparations when $CD8^+$ T lymphocytes were removed with magnetic beads (Fig. 4). In cells from two of the PtMs (Pt99P022 and Pt98P021), the kinetics and amounts of virus produced were similar to those seen for SIVmac239. It should be noted that NL-DT5R exhibited augmented replication in primary macaque cells compared with NL-ScaVR, the original nontissue culture-passaged construct. As expected, no replication of NL4-3 was detected in the $CD8^+$ T cell-depleted primary monkey cells.

Discussion

Our results are consistent with and extend numerous previously published single-cycle virus replication experiments that have reported species-specific APOBEC3G and TRIM5α restriction of HIV-1 in monkey cells. In our work, the establishment of spreading HIV-1 infections in simian cells represents an important step in significantly increasing the host range of HIV-1. It was conferred by inserting a 21-nucleotide SIV Gag CA element and the entire SIV *vif* gene into the genetic backbone of the

pNL4-3 HIV-1 molecular clone, plus four additional nucleotide changes acquired during long-term passage in a CyM lymphoid cell line. The proportion of HIV-1 sequences in the molecularly cloned NL-DT5R derivative obtained (93%) is substantially greater than that present in currently available CXCR4 (X4) using SHIVs (28–30%). It may be possible to increase the HIV-1 content of NL-DT5R further by mutating the DRMR amino acid residues at positions 14–17 of HIV-1 Vif to SEMQ, which is similar to the analogous region of SIV Vif. Such a change in the *vif* gene has recently been reported to confer replication competence to HIV-1 constructs in the presence of RhM APOBEC3G (31). Construction of other NL-DT5R variants bearing CCR5 using *env* genes from a variety of HIV-1 clades is also a future goal of these studies.

In contrast to commonly used X4 SHIVs, which carry SIV *gag* and *pol* genes, the NL-DT5R variant provides the opportunity to assess nonnucleoside RT inhibitors and a full spectrum of protease inhibitors, which specifically target HIV-1-, not SIV-, encoded enzymes. HIV-1 variants like NL-DT5R may also permit analyses of the cellular responses directed against HIV-1 Gag proteins that are associated with immunologic control and escape, not possible with currently available X4-tropic SHIVs.

Although the host range of the HIV-1 NL-DT5R derivative has expanded to include a monkey lymphoid cell line and CD8-depleted RhM and PtM PBMC, it still replicates less efficiently than SIV in simian cells. This observation undoubtedly reflects the high proportion (93%) of HIV-1 sequences present in the final construct, which have evolved for optimal replicative potential in human, not monkey cells. Additional changes will be required to achieve more robust infectivity for simian cells, which has already occurred to a limited extent with the acquisition of nucleotide substitutions after *in vitro* passaging. In addition to expected alterations in viral structural proteins, long-term passaging of NL-DT5R in monkeys may introduce changes in other HIV-1 sequences affecting analogous but subtly different SIV nonstructural proteins and cis-acting elements involved in processes such as transcriptional regulation and T cell activation pathways in monkey cells. Such alterations are likely to occur in the HIV-1 Nef protein, which is significantly smaller (205–210 aa long) than SIV Nef (260–265 aa long), and the HIV-1 LTR, which can be distinguished from its SIV analog by encoding: (*i*) different numbers/types of binding sites for transcriptional regulatory proteins and (*ii*) a single, not a double, stem-loop-bulge TAR element present at the 5′ termini of all viral transcripts (32). Although the replication properties of HIV-1 NL-DT5R in inoculated monkeys are presently unknown, they are very likely to be less robust than existing X4 SHIVs for the reasons noted above. Nonetheless, it is anticipated that extensive *in vivo* passaging of NL-DT5R will greatly augment its infectivity in macaque cells. In this regard, improved replicative and disease-inducing properties attended serial animal-to-animal transfers of first-generation nonpathogenic SHIVs, and they were associated with extensive sequence changes affecting multiple viral genes (33).

Materials and Methods

Construction of HIV-1 Proviral Clones. A pNL4-3-derived (34) vector, previously used for functional analyses of HIV-1 *vif* genes and designated pNL-SX (25), was the genetic backbone for the constructs shown in Fig. 1. In pNL-Sca, the 9-aa CypA-binding region of pNL-SX/NLVif (25) was replaced with the corresponding 7-residue segment from SIVmac239 CA by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To construct pNL-SV, the entire SIVmac *vif* sequence was amplified by PCR by using pMA239 (1) as template with forward TCC*CCCGGG*ATGGAGGAGGAAAAGAGGTGG and reverse GC*TCTAGA*TCATGCCAGTATTCCCAAGACC primers containing embedded SmaI and XbaI sites, respectively. The reactions were heated at 95°C for 5 min for 1 cycle; 95°C for 1 min, 51°C for 1 min, and 72°C for 1.5 min for 10 cycles; 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min for 25 cycles; and 72°C for 10 min for 1 cycle. The amplified product was subcloned into the SmaI–XbaI site of pNL-SX. To construct pNL-ScaV, the SmaI–XbaI fragment from pNL-SV was cloned into the equivalent sites of pNL-Sca. A negative-control clone, designated pNL-ScaV dBgl, contained a frameshift mutation at the BglII site of SIVmac239 *vif* in the pNL-ScaV.

Cell Culture. The 293T (human embryonic kidney), LLC-MKII (RhM kidney), Vero (AGM kidney), and OMK637 (owl monkey kidney) adherent cell lines were cultured in Eagle's MEM supplemented with 10% heat-inactivated FBS. A $CD4^+CXCR4^+CCR5^-$ CyM T cell line, HSC-F (27), was maintained in RPMI medium 1640 containing 10% FBS. RhM PBMCs were prepared and cultured as described previously (35). For PtM PBMC, a mixture of 95% Ficoll–Paque Plus (GE Healthcare, Piscataway, NJ) and 5% Dulbecco's modified PBS was used as a separation medium. To remove $CD8^+$ T cells from PBMCs, cells were stained with phycoerythrin (PE)-conjugated antihuman CD8 antibody (clone SK1; BD Bioscience, San Jose, CA), and then they were incubated with magnetic beads conjugated with anti-PE antibody (anti-PE MicroBeads; Miltenyi Biotec, Auburn, CA). Unstained cells were collected as the pass-through of a depletion column (LD Column, Miltenyi Biotec) according to the manufacturer's instructions.

Transfection, Infection, and RT Assays. Virus stocks were prepared by transfecting 293T cells with cloned HIV-1 NL4-3 derivatives by using either calcium phosphate coprecipitation (36) or Lipofectamine Plus (Invitrogen, Carlsbad, CA); 48 h later, culture supernatants were collected and stored at −80°C until use. Virion-associated RT activity was measured as described previously (28). HSC-F cells (1×10^7) were infected with equivalent amounts (1×10^7 RT units) of different virus preparations, and then they were monitored for RT activity in the culture supernatants. Macaque PBMCs (5×10^6) were infected with similar amounts (1×10^7 RT units) of the indicated viruses by spinoculation (30) for 1 h, and they were maintained for 12 days. The tissue culture medium was replaced daily.

Cloning of APOBEC3G Genes. Species-specific APOBEC3G cDNA was amplified from H9 (human), HSC-F (CyM), and Vero (AGM) cells by RT-PCR (described in *Supporting Methods*, which is published as supporting information on the PNAS web site) and cloned into pcDNA3.1-FLAG, an expression vector containing the FLAG tag sequence in pcDNA3.1 (Invitrogen). The expression levels of the three APOBEC3Gs in transfected 293T cells were monitored by immunoblotting using the anti-FLAG antibody.

Single-Cycle Replication Assays. The effects of the species-specific APOBEC3Gs on virus replication were evaluated by using VSV-G-pseudotyped HIV-1 stocks, prepared from 293T cells cotransfected with (*i*) individual *env*-deficient NL4-3 clones (NL4-3, NL-SVR, NL-ScaR, NL-ScaVR, and NL-ScaVR dBgl); (*ii*) pCMV-G (37), a VSV-G protein expression vector; and (*iii*) an individual species-specific APOBEC3G expression vector at a ratio of 8:1:1. The infectivity of the resultant viruses was determined by MAGI assay as described previously (38). To assess the effect of *gag* gene substitutions during single-cycle replication in cells from different primate species, VSV-G-pseudotyped viruses were prepared from 293T cells cotransfected with (*i*) individual *env*-deficient NL4-3 clones [NL4-3, NL-SVR, NL-ScaR, and NL-ScaVR] and (*ii*) pCMV-G, at a ratio of 9:1. Virus released into the medium and normalized for RT

activity was added directly or as 3-fold serial dilutions to 293T, LLC-MKII, and OMK637 cells, plated at a density of 5×10^4 cells per well in 24-well plates on the day before infection. On day 3 p.i., cells were lysed with CHAPS-based lysis buffer (28), and the amounts of intracellular p24 were determined by using the RETROtek p24 ELISA kit (ZeptoMetrix, Buffalo, NY). The total amount of protein in each sample was determined in parallel with the DC protein assay kit (Bio-Rad, Hercules, CA) to normalize for different cell-harvesting efficiencies.

Generation of pNL-DT5. HSC-F cells were infected NL-ScaV virus prepared from transfected 293T cells as described above. Half of the culture medium (5 ml) was replaced every 3 days, and the harvested supernatants were stored at −80°C. Fresh HSC-F cells (1×10^7) were added on days 27, 36, and 45 p.i., and the culture was maintained until 51 days p.i. The supernatants collected on days of 39 and 51 p.i. were filtered through a 0.45-μm filter and used to initiate a second round of infection (5×10^6 RT units of viruses added to 1×10^7 HSC-F cells). On day 18 p.i., cells infected with the day 51 supernatant (sup 51) were collected (Fig. 3*C*), and the integrated provirus was amplified from genomic DNA as two overlapping fragments by DNA PCR. The 5′ fragment extended from 5′ LTR to the Vpr-coding region, whereas the 3′ fragment spanned the Vif-coding region to the 3′ LTR. The 5′ fragment was amplified with the NL1–24Aat-5′ (AGTCA*GACGTC*TGGAAGGGCTAATTTGGTCCCAAA at nucleotide positions 1–24 in NL4-3) and NL5832–5855Bam-3′ (ATCGCGGATCCTCTAGTCTAGGATCTACTGGCTCC at 5832–5855) primer pairs, whereas the 3′ fragment was amplified with the NL5596–5619Xba-5′ (GCTAGTCTAGAAGCCATACAATGAATGGACACTAG at 5596–5619) and NL9686–9709Sph-3′ (ACATG*GCATGC*TGCTAGAGATTTTCCACACTGACT at positions 9686–9709) primer pairs. The reactions were heated at 95°C for 5 min for 1 cycle; 95°C for 0.5 min, 51°C for 0.5 min, and 72°C for 6 min for 10 cycles; 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 6 min for 25 cycles; and 72°C for 10 min for 1 cycle. The amplified 5′ and 3′ viral DNA segments were digested with AatII–EcoRI and EcoRI–SphI, respectively, and they were then cloned together into pUC19 digested with AatII–SphI. The resultant proviral clone was designated pNL-DT5.

This work was supported in part by Research Fellowship for Young Scientists 17-0561 (to K.K.) and Grant-in-Aid for Scientific Research B 18390140 from the Japan Society for the Promotion of Science (to A.A.) and by Health Sciences Research on HIV/AIDS Grant 16150301 from the Ministry of Health, Labor, and Welfare of Japan. This work was also supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Effects of lysine to arginine mutations in HIV-1 Vif on its expression and viral infectivity

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Received May 17, 2006; Accepted July 5, 2006

Abstract. We previously demonstrated that the expression in cells of human immunodeficiency virus type 1 (HIV-1) Vif is maintained at low level by proteasome-degradation. We examined the contribution of 16 lysines present in Vif (NL432 clone), which is composed of 192 amino acids (aa), to its expression within cells and to viral infectivity for non-permissive cells. To this end, various lysine-arginine mutations were introduced into wild-type (wt) Vif, and the mutational effects were monitored by transfection experiments. When all the lysines were changed to arginines, the mutant Vif was expressed in cells at much higher level than wt and was much more stable. Both N-terminal (aa nos. 34 and 36) and C-terminal (aa nos. 179 and 181) lysines were found to be almost sufficient for wt property. Different from this observation, one of the lysines at aa nos. 22 and 26 was demonstrated to be essential for the virus to grow in non-permissive cells. Our results showed that there is no clear co-relationship between the expression level of HIV-1 Vif and viral infectivity.

Introduction

Vif is one of the human immunodeficiency virus type 1 (HIV-1) accessory proteins, and is conserved in all known primate immunodeficiency viruses (1). It is dispensable for the replication of HIV-1 in permissive cells like MT-4 (2) and M8166 (3), but is critical for the viral growth in non-permissive cells such as H9 (4) and peripheral blood mononuclear cells (5-9). Recent studies have shown that the non-permissive cells have a cytidine deaminase APOBEC3G carrying anti-viral activity, and that the Vif counteracts the virion incorporation of APOBEC3G (10-19). The precise molecular mechanism for this activity of Vif, however, remains to be elucidated.

We have recently demonstrated that the expression of HIV-1 Vif is controlled uniquely to be at low level among accessory proteins by proteasome degradation (20,21). Virological significance of this degradation can be explained by the fact that a high expression level of Vif inhibits viral infectivity through modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site (22). However, the experiments were done in a quite artificial system; expression of a large amount of Vif by pNL-A1 (23) and examination of infectivity by a single-round replication assay.

In this study, we investigated the relationship between the Vif expression level within cells and viral multi-cycle infectivity for the non-permissive cells. For this purpose, we introduced a wide variety of lysine to arginine mutations into wild-type (wt) Vif, because it is well known that proteins are poly-ubiquitinated at their lysine residues or N-terminus to become a marker recognized by the proteasome, and that the polyubiquitinated proteins are then degraded (24); the lysine and arginine have similar physicochemical characteristics. We identified lysines in Vif which are important for the wt expression level within cells and for the viral multi-cycle infectivity in non-permissive cells.

Materials and methods

Cells. A lymphocytic cell line H9 (4) was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). A monolayer cell line 293T (25) was cultured in Eagle's minimal essential medium supplemented with 10% heat-inactivated FBS.

Transfection. For transfection of plasmid DNAs into adherent 293T cells, the calcium-phosphate coprecipitation technique (26) or the Lipofectamine Plus™ system (Invitrogen, Carlsbad, CA, USA) was used. For transfection of lymphocytic H9 cells, the electroporation method was used as previously described (26).

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Key words: HIV-1, Vif, lysine, arginine

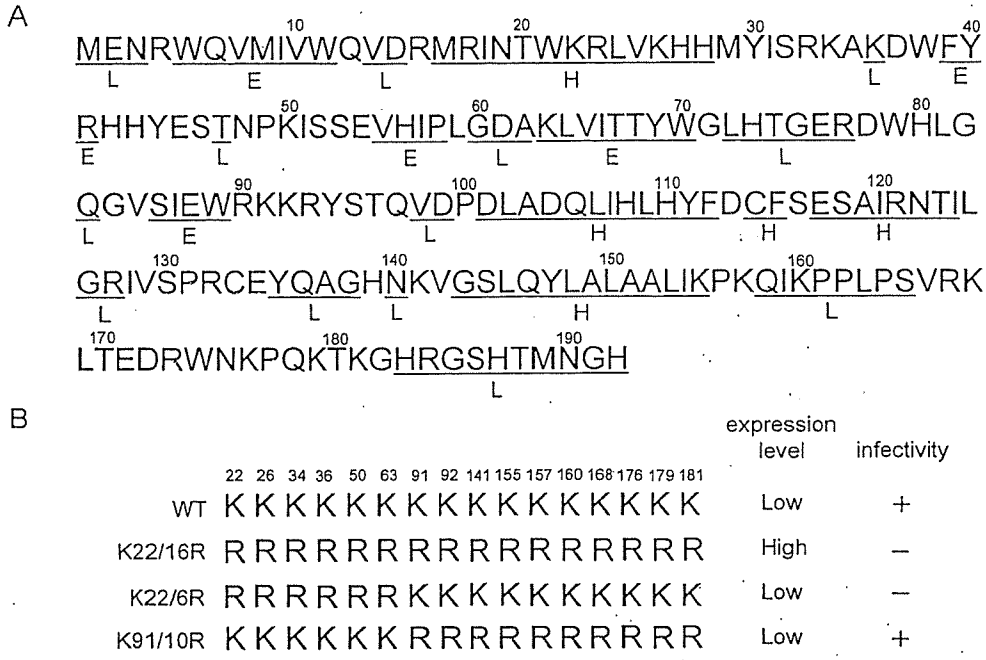

Figure 1. The lysine-arginine alterations in the first group of Vif mutants. Structure of the Vif of HIV-1 NL432 clone by the PredictProtein (http://www.predictprotein.org/) (A) and the alterations in this Vif (the first group mutants) (B) are indicated. L, E and H in (A) represent the loop, ß-strand and α-helix structures, respectively. Data on the expression level in cells of the mutated Vif proteins and the infectivity for H9 cells of the mutants are also shown in (B) (see Figure 2).

Reverse transcription (RT) assay. Virus production in the culture supernatants of transfected H9 cells was monitored by RT assay as previously described (27).

Western immunoblot analysis. Transfected 293T cells were collected and solubilized by dissolving in PBS-Laemmli's sample buffer (1:1) for SDS-PAGE as previously described (20). Samples resolved by the SDS-PAGE were then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were treated with anti-FLAG antibody (Ab) (ANTI-FLAG M2 Monoclonal Ab, Sigma-Aldrich, St. Louis, MO, USA) and visualized using the ECL plus Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Pulse/chase analysis. Transfected 293T cells were pulse-labelled with ^{35}S, and chased as previously described (20). Cells harvested were lysed with the CHAPS/DOC buffer as described previously (20), and the cell lysates were precipitated with a Vif-specific polyclonal antibody Vif93 (28). Wt and mutant Vif proteins were identified by SDS-PAGE followed by fluorography as previously described (20).

DNA constructs for infection experiments. An infectious proviral clone of HIV-1 designated pNL432 (26) was used as wt clone for infection experiments. Appropriate fragments of pNL432 were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA, USA) and mutations were introduced. The alterations of lysines into arginines were performed by the QuikChange site-directed mutagenesis kit (Stratagene). The mutated fragments were cloned back to wt to construct pNL-K22/6R, pNL-K91/10R, pNL-K22/2R, pNL-K34/2R, pNL-K50R, pNL-K63R, pNL-K22R and pNL-K26R. To make pNL-K22/16R, appropriate fragments of pNL-K22/6R and pNL-K91/10R were used. Clone pNL-K34/14R was constructed from the pNL-K22/16R similarly as above. As a negative control, pNL-Nd (29) carrying a frame-shift mutation in *vif* was used.

DNA constructs for Western immunoblot and pulse/chase analyses. The pNL-A1S (21) was used to construct expression vectors for Western blot and pulse/chase analyses. To generate pNL-ASCF, a *Cla*I site and the FLAG sequence (in this order) were introduced just upstream of the stop codon of *vif* in pNL-A1S (21) by the QuikChange site-directed mutagenesis kit. The *vif* sequences of pNL432 and its mutants, pNL-K22/16R, pNL-K22/6R, pNL-K91/10R and pNL-K34/14R, were amplified by polymerase chain reaction (PCR) with *Sma*I at 5' and *Cla*I at 3' ends, respectively. The *Sma*I-*Cla*I fragment from the pNL-ASCF was replaced with these PCR-amplified sequences to construct pNL-ASCF-fWT, pNL-ASCF-fK22/16R, pNL-ASCF-fK22/6R, pNL-ASCF-fK91/10R and pNL-ASCF-fK34/14R. An appropriate fragment of pNL-ASCF-fK22/16R was subcloned into pBluescript SK(+), and mutations were introduced by the QuikChange site-directed mutagenesis kit. The mutated fragments were cloned back to pNL-ASCF-fK22/16R to construct pNL-ASCF-K22/2R/50/12R, pNL-ASCF-K22/6R/141/8R, pNL-ASCF-K22/9R/160/5R and pNL-ASCF-K22/14R. As an expression vector for luciferase, pGL3-Control Vector (Promega, Madison, WI, USA) was used.

Results

Expression and infectivity of various HIV-1 Vif mutants. We examined the importance of lysines present in Vif for its

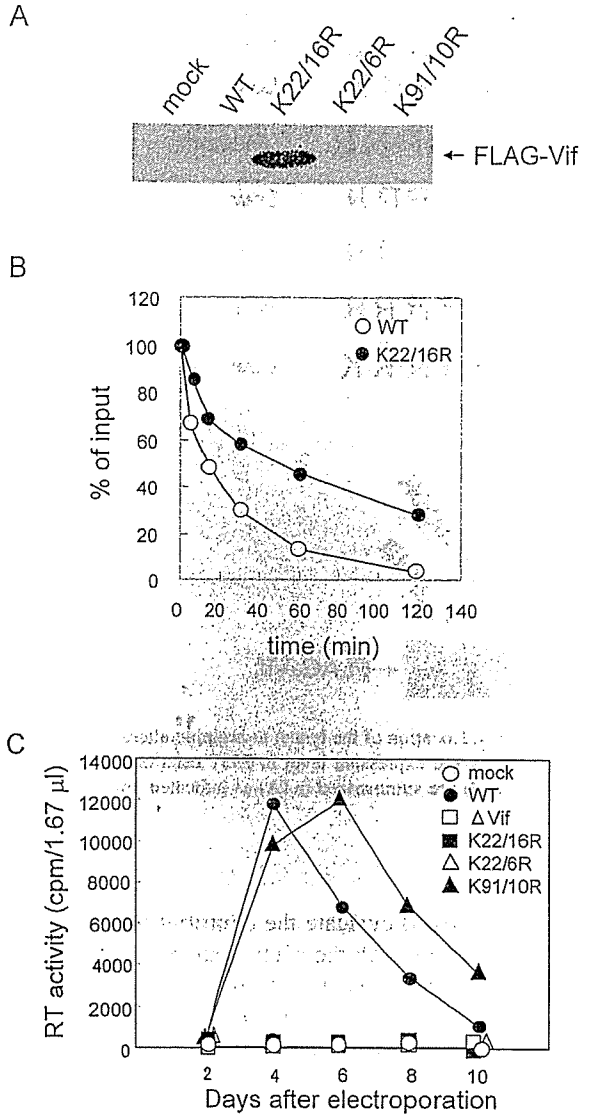

Figure 2. Characteristics of the first group of Vif mutants. (A) Expression level in cells of wt and mutant clones. Co-transfected 293T cells with 7.5 μg of the subgenomic viral clones derived from pNL-ASCF-fWT and 2.5 μg of an expression vector for luciferase, at 48-h post-transfection, were harvested for Western blot analysis and luciferase assay. The loading amount in each lane was normalized by the luciferase activity. mock, pUC19; WT, pNL-ASCF-fWT. (B) Degradation kinetics in 293T cells of the K22/16R mutant. Transfected 293T cells with 5 μg of the subgenomic clones, and at 24-h post-transfection, were collected, ^{35}S-labeled, and chased for ≤120 min for immunoprecipitation analysis. WT, pNL-ASCF-fWT. (C) Growth kinetics in non-permissive cells of wt and mutant clones. H9 cells were transfected with 10 μg of the full-length viral clones, and virus production in the culture supernatants was monitored by RT assay. mock, pUC19; WT, pNL432; ΔVif, a frame-shift mutant pNL-Nd.

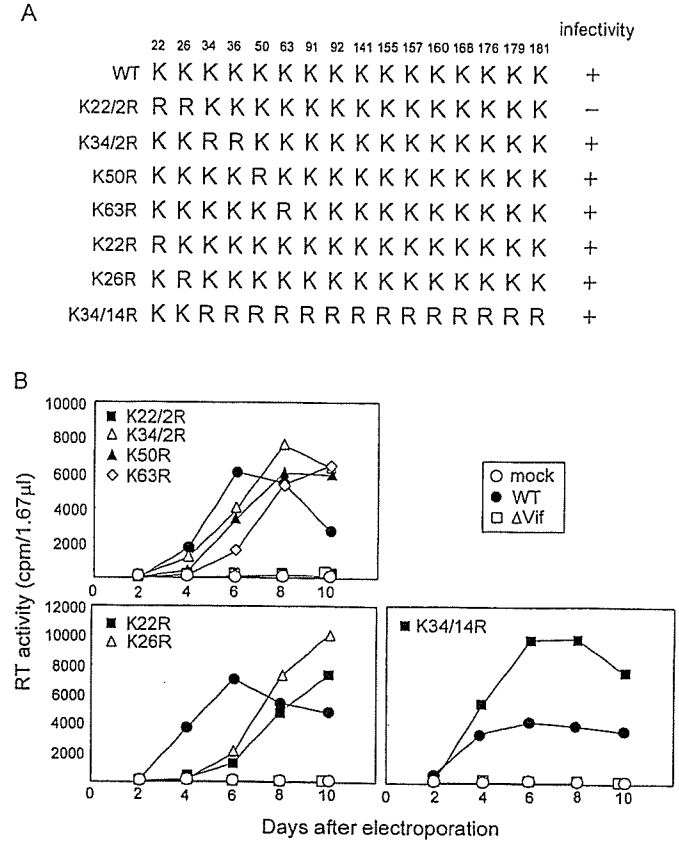

Figure 3. Identification of the lysine residue in Vif critical for viral infectivity. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their growth kinetics in H9 cells (B) are shown. For determination of viral infectivity in H9 cells, cells were transfected with various full-length clones and monitored for virus production as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL432; ΔVif, a frame-shift mutant pNL-Nd.

expression and viral infectivity. As the first group mutants for this study, clones K22/16, K22/6R and K91/10R, which carry all 16, N-terminal 6 and C-terminal 10 lysine-ariginine exchanges, respectively, were constructed and characterized (Fig. 1). For a quantitative comparison, their expression to a high level was achieved by the subgenomic-type clone (21) and analyzed by Western immunoblotting using anti-FLAG antibody. As shown in Fig. 2A, the mutant K22/16R produced its Vif at an extremely high level relative to those of wt, K22/6R and K91/10R. We determined the stability of the K22/16R Vif by the pulse/chase experiment as previously described (20). As shown in Fig. 2B, in a good agreement with the steady-state expression level, K22/16R was much more stable than wt. We next examined the infectivity of these mutants for non-permissive cells. H9 cells were electroporated with the full-length version of the mutant clones, and virus growth was monitored by RT assay (30). As shown in Fig. 2C, only the mutant K91/10R among the three mutants, which express a high or negligible level of Vif in cells (Fig. 2A), grew fairly well.

In total, our results described herein indicated that the lysines present in Vif were important for the stable expression of Vif, and that there is no clear negative or positive co-relationship between the expression level of Vif and infectivity of lysine-ariginine mutants.

Lysine residues in Vif important for viral infectivity. To determine the lysine residue in Vif crucial for viral infectivity, we constructed another set of proviral mutant clones. One or two lysines located at the N-terminal region of Vif were changed to the arginine residue (K22/2R, K34/2R, K50R and K63R in Fig. 3A), and the resultant clones were examined for their growth kinetics in H9 cells as above. As shown in Fig. 3B, only the K22/2R were not infectious. Therefore, we

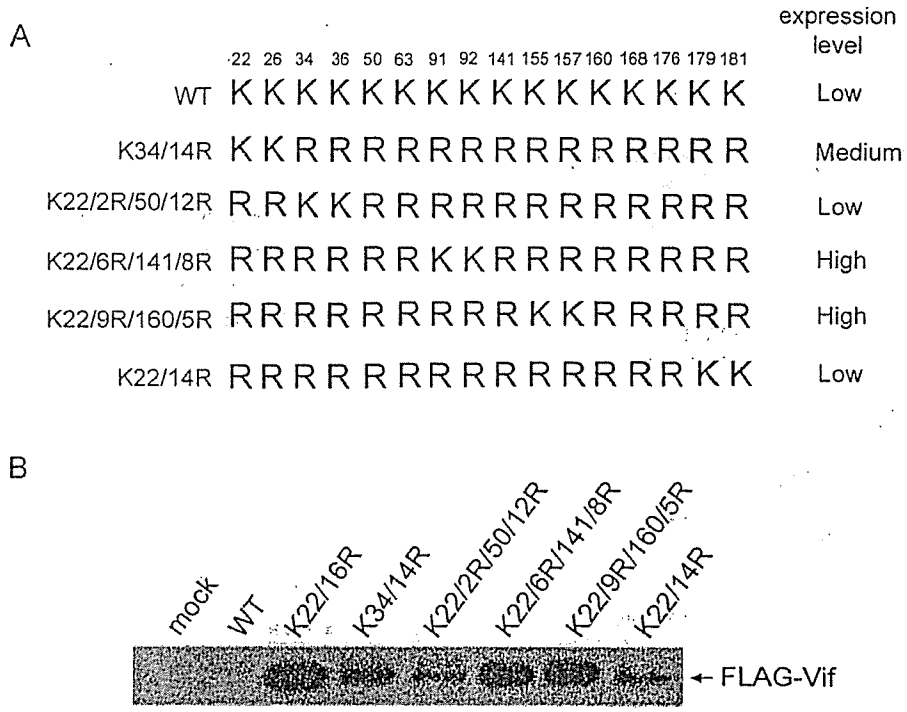

Figure 4. Identification of the lysine residue in Vif critical for the wt expression level within cells. Location of the lysine to arginine alterations in the Vif of the mutants (A) and their expression in the transfected 293T cells (B) are shown. For monitoring the expression level in 293T cells of the Vif, cells were transfected with various subgenomic clones and analyzed for their expression as above. Data in (B) are summarized in (A) as indicated. mock, pUC19; WT, pNL-ASCF-fWT.

constructed K22R, K26R and K34/14R (Fig. 3A) to determine whether one of the K^{22} and K^{26} or both are essential for viral infectivity, and whether the lysines other than K^{22} and K^{26} are critical for viral infectivity. The three additional mutants thus constructed were transfected into H9 cells, and their growth was examined. As shown in Fig. 3B, the three mutants grew to a comparable extent. All the mutants described above were confirmed to propagate in permissive M8166 cells (data not shown). Collectively, we concluded that either K^{22} or K^{26} of Vif is critical for the productive infection of HIV-1 NL432 in non-permissive cells.

Lysine residues in Vif important for the expression level of Vif. To determine the lysine residue in Vif crucial for the Vif expression, we constructed a series of double lysine-arginine mutants other than the K34/14R, based on the subgenomic clone (21) (Fig. 4A). The mutants were then analyzed for their Vif expression by Western immunoblotting as described above. When the mutant K34/14R, which is infectious for H9 cells (Fig. 3B), was monitored for its Vif expression, it displayed a medium expression level between wt and the stable and non-infectious mutant K22/16R (Fig. 4B). This observation again indicated the absence of a detectable close relationship between viral infectivity and the Vif expression level. The data on the other mutants in Fig. 4B clearly showed that the K^{34} and K^{36} or the K^{179} and K^{181} are responsible for the low expression level of Vif. While the mutants K22/6R/141/8R and K22/9R/160/5R produced a similarly high expression level to that of the K22/16R, the mutants K22/2R/50/12R and K22/14R expressed a low level of Vif quite similar to the wt clone. We also constructed a complete set of single lysine-arginine mutants through one by one alterations to evaluate the contribution of each lysine to the Vif expression. All the single mutants constructed were found to produce Vif, upon transfection, at a level higher than that of the mutants K22/2R/50/12R and K22/14R (data not shown). These results strongly suggested that each lysine residue in Vif is less important than the combination of K^{34} and K^{36} or of K^{179} and K^{181} for the wt expression level of Vif.

Discussion

In this study, we showed by a mutational analysis that the lysines in HIV-1 Vif are important for its steady-state expression in transfected 293T cells, and that the two lysines in Vif (K^{34} and K^{36} or K^{179} and K^{181}) are nearly sufficient for the expression property of the Vif (Figs. 2 and 4). We also demonstrated that either K^{22} or K^{26} in Vif is critical for the replication of HIV-1 in H9 cells (Figs. 2 and 3). Thus, we did not find any clear co-relationship between the expression level in cells of Vif and viral infectivity for the non-permissive cells.

Together with the results previously published (20,22), it was reasonable to assume that the K^{34} and K^{36} or K^{179} and K^{181} are important to maintain the low appropriate expression level of Vif by the proteasome-degradation, and thereby enable the virus to grow in non-permissive cells. However, our data herein on the mutants demonstrating that either K^{22} or K^{26} is critical for viral infectivity do not support this prediction, and instead, the mechanism(s) and molecule(s) other than the proteasome-degradation and its associated factors which confer the infectivity on the virus should be considered. They would include 1) the structure of the lysine itself, 2) covalent modification(s) of the lysine and 3) interaction of the lysine

with some unknown factor(s). Further study is required to clarify the molecular basis for our observations on the Vif mutants.

Acknowledgements

We thank Ms. Kazuko Yoshida for editorial assistance. This work was supported by a Grant-in-Aid for Scientific Research (B) (18390140) and a Grant-in-Aid for Scientific Research (C) (15590420) from the Japan Society for the Promotion of Science, and a Health Sciences Research Grant (Research on HIV/AIDS 16150301) from the Ministry of Health, Labor and Welfare of Japan. B.K. is supported by the Royal Thai Government Scholarship 2003 (Commission on Higher Education).

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