HIV-1 capsid mutants inhibit the replication of wild-type virus at both early and late infection phases

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Abstract In-frame mutations were introduced into various portions of the human immunodeficiency virus type 1 (HIV-1) *gag* gene, and potentials of the mutants to suppress the replication of wild-type HIV-1 were monitored. In contrast to results obtained with matrix and nucleocapsid mutants, almost all capsid mutants blocked HIV-1 replication completely in single-round replication assays. A capsid mutant designated C6b was demonstrated to be one of the most efficient inhibitors for HIV-1 reported to date, and to be effective at both early and late viral replication phases. T-cells, which are engineered to express the C6b Gag in response to HIV-1 infection, were perfectly resistant to HIV-1.

Key words: Human immunodeficiency virus type 1; Gag; Capsid; Matrix; Nucleocapsid

1. Introduction

Mutations can confer a dominant negative phenotype on viruses. When a gene carrying such a mutation is expressed, its product can dominantly interfere with the function normally accomplished by the product of the wild-type (wt) gene. When the gene product is multimeric, an effective dominant negative mutant could be one that makes a monomer still capable of interacting with wt protein but is otherwise defective, and that could recruit wt monomers into non-functional multimers. In such a case, only a moderate level of expression of the dominant negative mutant would be sufficient to exert a strong inhibitory effect on the wt protein. At least three distinct HIV-1 genes, *tat*, *rev*, and *gag*, are potential targets for the generation of dominant negative mutants. In fact, dominant negative mutants of Tat, Rev, and Gag have been described [1–4]. Because of the highly multimerized state of Gag proteins in the mature virion, dominant negative mutants of Gag may be the most effective. However, a systemic analysis of *gag* gene mutants with respect to their ability to interfere with the replication of wt virus and to the molecular basis underlying this interference has not yet been carried out. Furthermore, no quantitative data on the efficiency of the inhibitory effect have been reported.

To investigate the functions of HIV-1 Gag proteins, we have recently constructed a series of *gag* mutants by linker insertions. All 20 mutants generated carry in-frame mutations, and the mutations are scattering in the *gag* gene. Phenotypic characterization showed that these mutants are defective at various stages of the viral replication cycle (our unpublished results). Thus, our Gag mutants were good candidates to monitor the *trans*-dominant negative property. For facilitation of this analysis, the single-round replication assay recently developed by us [5,6] was used. In this report, we present data on systemic and quantitative analysis of the interfering potentials of the HIV-1 capsid, matrix, and nucleocapsid mutants. We also show the viral replication phase where the interference occurred.

2. Materials and methods

2.1. Cell culture and DNA transfection

Human CD4+ lymphoid cell lines, A3.01 [7], CEMx174 [8], and U937 [9], were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). A human colon carcinoma cell line, SW480 [10], was maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS. For transfection, uncleaved plasmid DNA was introduced into SW480 and lymphoid cells by the calcium phosphate co-precipitation [10] and modified DEAE-dextran [11] methods, respectively. To obtain G418-resistant stable lines, CEMx174 cells were transfected with DNAs by the electroporation method [12].

2.2. CAT and RT assays

The chloramphenicol acetyltransferase (CAT) and reverse transcriptase assays have been previously described [13,14].

2.3. Single-round replication assay

The process of HIV-1 replication was quantitatively analyzed by a system designated single-round replication assay (SRA) [5,6].

2.4. Western blotting

Western immunoblotting was performed as previously described [14]. Specific viral Gag proteins were detected by the ECL system using a monoclonal antibody against HIV-1 Gag p24 (Amersham).

2.5. DNA constructs

An infectious molecular clone of HIV-1 designated pNL432 has been described previously [10]. All in-frame *gag* gene mutants listed in Fig. 1 and Table 1 were constructed from pNL432 by linker insertions. Mutations introduced into pNL432 were confirmed by nucleotide sequencing. Rev-inducible Gag expression vectors designated pRgWT (wt) and pRgC6b (mutant) were constructed by cloning their parental gag (*Bss*HII-*Hin*cII) and RRE (rev-responsive element) (*Nhe*I-*Bam*HI) sequences into pRVSVneo [15]. A Rev expression vector designated prev1 was previously described [5,6].

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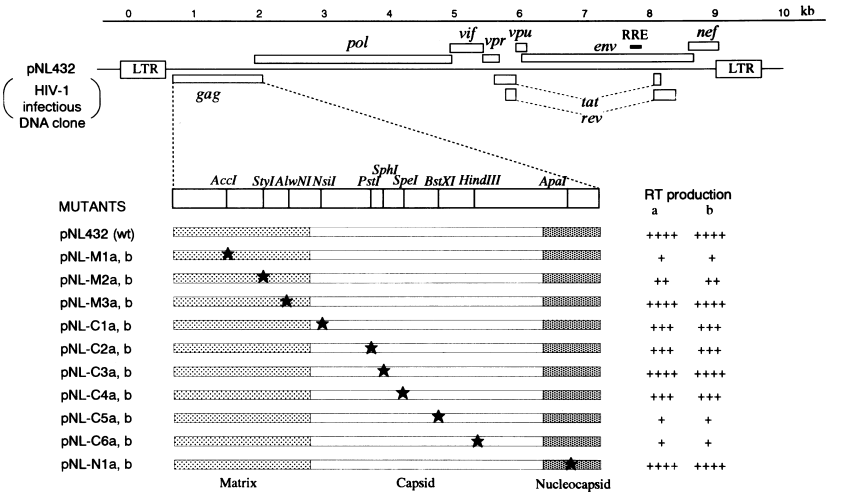

Fig. 1. In-frame *gag* gene mutants of HIV-1 used in this study. Sites of mutations introduced are indicated by ★. Small letters a and b indicate that *Not*I and *Cla*I linkers (Takara), respectively, were used for mutagenesis except for C6b (*Xho*I linker). RT production in SW480 cells at 48 h after transfection of each mutant (20 μg) is shown on the right. ++++, 90–110% of wt; +++, 50–70%; ++, 20–40%; +, < 10%.

3. Results

3.1. Generation of gag gene mutants

A total of 20 *gag* gene mutants were constructed from pNL432 (Fig. 1 and Table 1). We employed scattered mutagenesis by linker insertions (Fig. 1), and all mutations were in-frame (Table 1) as confirmed by nucleotide sequencing. The resultant mutants contained insertions of two to four amino acids, and some bore, in addition, substitutions of amino acids (Table 1). When transfected into SW480 cells [10], the mutants generated progeny virions as monitored by RT assay to varying degrees (Fig. 1). The effects of mutations were dependent on the positions of mutations, but not on the linkers used (thus, not on the amino acids). It appeared that the N-terminal portion of matrix p17 and the C-terminal portion of capsid p24 are important for virion production (Fig. 1).

3.2. Potentials of the gag mutants to suppress the replication of wt clone

We recently developed a system designated SRA [5,6] for determination of the defective step of HIV-1 mutant. This system utilizes replication-defective *rev*(−) proviral clones carrying CAT (pNLnCAT etc.) and is dependent on *trans*-complementation for production of infectious vector virus [5,6]. Simply by monitoring CAT activity in cells infected with this CAT virus, one round of viral replication cycle can be quantitatively estimated [5,6]. In this study, proviral wt and *gag* mutant clones instead of prev1 [5,6] were used as a Rev supplier.

SW480 cells were co-transfected with pNLnCAT and wt or mutant clones, and supernatants were harvested 2 days later. Equal volumes of the supernatants were inoculated into CD4+ A3.01 cells, and 3 days later, cells were harvested for the CAT assay. As shown in Fig. 2, the wt sample generated a very high level of CAT activity. A fair level of CAT was induced in cells infected with most of the matrix and nucleotide mutants. In contrast, all capsid mutants, with one exception, inhibited CAT production almost completely. Essentially the same results were obtained in another lymphocytic cell line, CEMx174, and a monocytic cell line, U937 (data not shown). One of the mutants with a high ability of inhibition, designated NL-C6b, was tested for its ability to interfere with

Table 1
Amino acid sequences of *gag* gene mutants of HIV-1 NL432 used in this study

Mutants		Mutants	
	(57)C----R(58)		(218)VHA--G(221)
M1a	CRAAAR	C3a	VSGRSG
M1b	CSGRPR	C3b	VPSMAG
	(98)K----E(99)		(240)T----S(241)
M2a	KAAAKE	C4a	TRRPPS
M2b	KHRCKE	C4b	TSIDAS
	(118)AAA---D(121)		(280)TSI--L(283)
M3a	AVAAATD	C5a	TRAAAL
M3b	AAHRWAD	C5b	TSHRWL
	(152)LNA--W(155)		(309)A----S(310)
C1a	LKAAAW	C6a	AAAAAS
C1b	LNHRWW	C6b	APRGAS
	(209)AA--E(211)		(405)CRA--P(408)
C2a	AAAAE	N1a	CRAAAP
C2b	AHRWE	N1b	CSHRWP

WT sequences are shown on the upper line. Figures in parentheses represent the amino acid numbers of NL432 Gag protein.

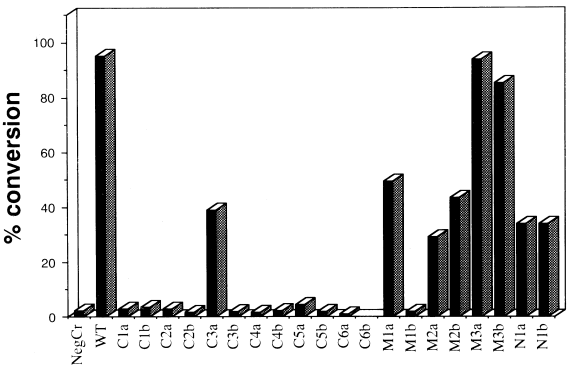

Fig. 2. Inhibition of HIV-1 replication as monitored by SRA. SW480 cells were cotransfected with pNLnCAT (10 μg) and pNL432 or mutant clones (10 μg), and 2 days later, culture supernatants were harvested. Equal volumes of the supernatants were inoculated into CD4+ A3.01 cells, and 3 days later, cells were harvested for CAT assay. The parental pNL432 and pUC19 were used as positive (WT) and negative (NegCR) controls, respectively.

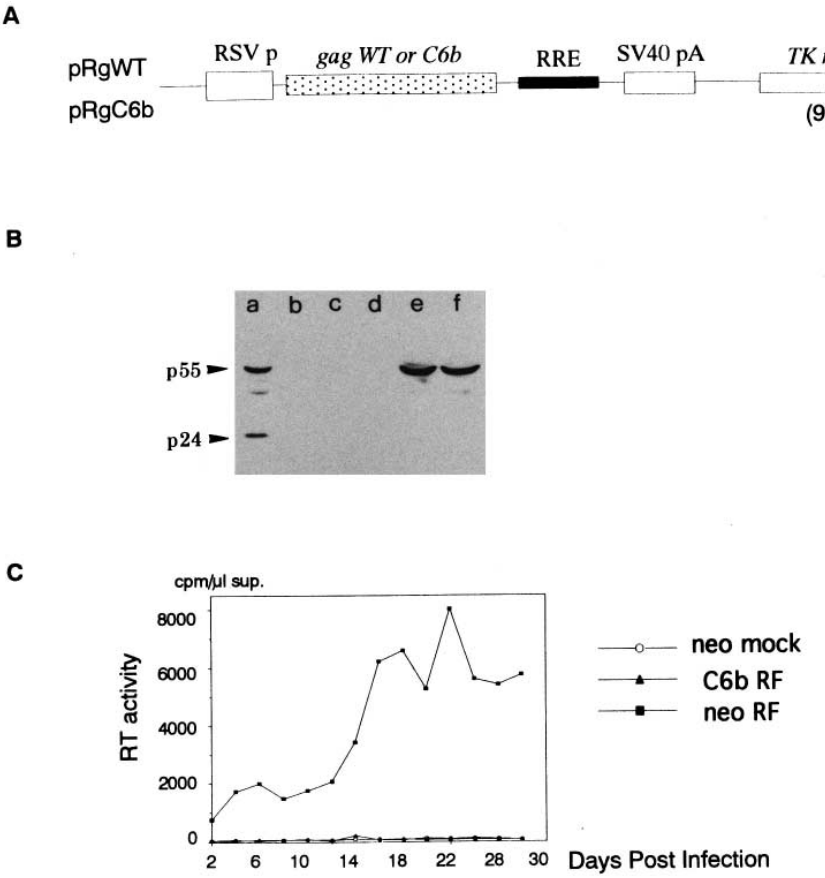

Fig. 3. Expression of C6b in cells. A: Structure of Gag expression vector. The *gag* sequence is flanked by RSV promoter (RSVp) and SV40 poly(A) signal (SV40pA). RRE sequence is placed between *gag* and SV40pA. Another expression unit of *neo* gene is present in the vector [15]. B: Western blotting analysis of cell lysates prepared from transfected SW480 cells. SW480 cells were transfected with pNL432 (a), pRgWT (c), pRgC6b (d), pRgWT plus prev1 (e), or pRgC6b plus prev1 (f); b, mock transfection. A monoclonal antibody against HIV-1 p24 was used to detect viral Gag proteins. The bands were visualized by the ECL system (Amersham). HIV-1 *gag* gene products p55 and p24 are indicated. C: Infection of G418-resistant CEMx174 cells with HIV-1. CD4+ CEMx174 cells were transfected with pRVSVneo (control) or pRgC6b, and cultured in the presence of G418. Surviving cells were maintained and used for infection of HIV-1 RF strain. Only cells of positive control were productively infected with the RF strain (neo RF). Cells of negative control (mock infection; neo mock) and C6b(+) cells (C6b RF) gave no detectable virus after infection of HIV-1 RF.

the replication of the wt clone in more detail. A dose-response relationship with regard to inhibition of the wt replication was determined by the same method described in Fig. 2. In the presence of more C6b than wt by only twofold, the replication of wt clone was actually completely blocked (data not shown).

We next asked whether C6b can suppress the growth of wt HIV-1 in CD4 cells. For this purpose, Gag expression vectors designated pRgWT and pRgC6b were constructed (Fig. 3). The expression of the constructs was designed to be Rev-inducible (Fig. 3A), since Gag might be more or less toxic for cells and should not be expressed without HIV infection. As clearly seen in Fig. 3B, Gag p55 was expressed only in the presence of Rev, and was really Rev-inducible. Cells which contain the C6b sequence in the genome were selected by G418 after electroporation of pRgC6b. The cells obtained were then infected with HIV-1 RF strain, and monitored for virus infection. Neither virus production nor viral CPE was observed (Fig. 3C).

3.3. Stage in the viral replication cycle of the C6b mutant acting as a repressor

The critical phase of viral replication when C6b is effective for inhibition was determined by the SRA using pNLnCAT (proviral *rev*(−) reporter CAT construct) [5,6], prev1 (Rev expression vector) [5,6], pRgWT, and pRgC6b. As shown in Fig. 4A, no interference by C6b was detected in the process of transcription and translation as monitored by the first CAT assay [5,6]. In sharp contrast, virion production as monitored by the RT assay [5,6], and the proceeding of the early replication phase as monitored by the second CAT assay (from entry to DNA integration) [5,6], were greatly affected (Fig. 4B,C). These inhibitory effects of C6b on the replication of the wt clone could account for the lack of productive viral infection in C6b(+) cells (Fig. 3C).

4. Discussion

In this study, we evaluated the ability of 20 *gag* gene mutants to interfere with the replication of wt HIV-1. We demonstrate here that the capsid mutants are very effective against HIV-1 replication (Fig. 2). More importantly, the observed inhibition was very efficient, and C6b would be better than the other *trans*-dominant negative mutants previously reported [1–3]. Of note is that a mass culture of cells, which had been transfected with pRgC6b, was perfectly resistant to HIV-1 infection. Without selection of particular cell clones

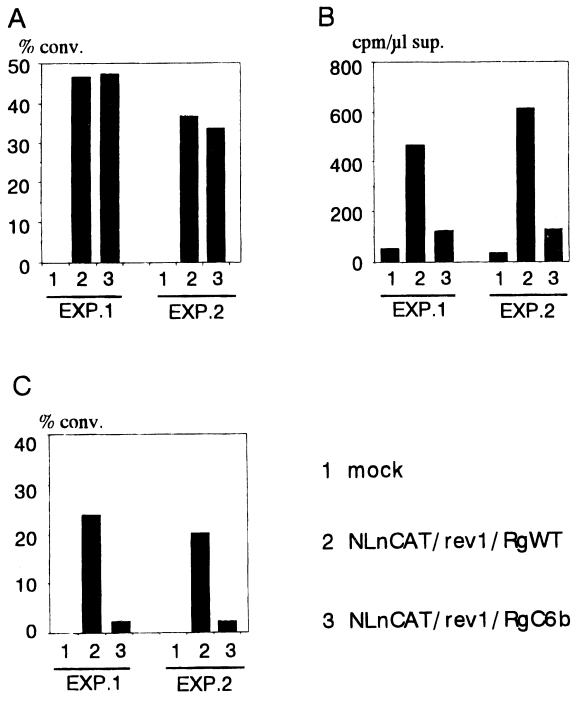

Fig. 4. Effective inhibitory stage in viral replication cycle of C6b. All the data presented were obtained by SRA described previously [5,6]. CAT (A) and RT (B) production in transfected cells is indicative of a normal proceeding of the late replication phase (from transcription and translation to virion release). The early phase of viral replication (from entry to viral DNA integration) can be monitored by measuring CAT activity (C) in cells infected with equal amounts of the CAT virus (1×10^5 RT units) obtained by transfection (B). CD4+ A3.01 cells were used throughout the experiments here. DNA clones used in (A) and (B), and viruses obtained from transfection and used in (C) are indicated on the lower right.

which are highly resistant to the virus, no virus production was detected (Fig. 3C). Furthermore, C6b(+) cells would express the mutant Gag only after HIV-1 infection.

The C6b Gag interfered with HIV-1 replication at both early and late phases (Fig. 4). Although the precise mechanism underlying this result remains to be elucidated, it is quite reasonable to assume that wt and C6b Gag assemble into virions poorly, and that chimeric virions consisting of wt and C6b Gag are very ineffective to initiate infection. It is also possible that the late defect of the NL-C6b virus is the production of immature virions with poor RT activity. Our preliminary data suggest that the early defect of NL-C6b virus could be located at the process of uncoating/reverse transcription. Further study is required to fully understand the function of HIV-1 Gag.

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