Cleavage of Gag precursor is required for early replication phase of HIV-1

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Abstract A mutant of human immunodeficiency virus type 1 (HIV-1), which is deficient for Gag precursor cleavage and non-infectious, was characterized with respect to its defective step in the viral replication phase. Upon transfection, the mutant produced a normal level of progeny virions as monitored by electron microscopy and RNA hybridization. Single-round replication assay demonstrated, in contrast, that the mutant was defective at the early phase of the replication cycle. Furthermore, no viral DNA was detected in the cells infected with the mutant. Taken together, it is concluded that maturation of Gag precursor protein of HIV-1 is required for an early event(s) before or during a coupled process of uncoating/reverse transcription.

Key words: Human immunodeficiency virus type 1; Protease; Gag; Uncoating; Reverse transcription

1. Introduction

The functional importance of human immunodeficiency virus type 1 (HIV-1) protease for viral replication has been well documented [1–3]. In HIV-1, structural and enzymatic components of the virion are expressed as polyprotein precursors, i.e. Gag ($p55^{gag}$) and Gag-Pol ($p160^{gag-pol}$)[4], respectively, which assemble into immature virions on the cytoplasmic side of the cell membrane [1]. The HIV-1 protease is part of the $p160^{gag-pol}$. During and after virion budding from the cell membrane, proteolytic processing of the $p55^{gag}$ and $p160^{gag-pol}$ polyprotein precursors is initiated by the viral protease, leading to mature Gag and Pol proteins, condensation of the viral core, and hence, infectious virions [1–3]. HIV-1 can assemble in the absence of functional viral protease, but the resultant virions are non-infectious and morphologically aberrant [1–3,5].

Although it is apparent that the HIV-1 protease functions at the late viral replication phase for maturation of progeny virions, it is not clear whether the protease is required for efficient release of virions from cells, and furthermore, the molecular basis for the non-infectivity of virions produced in the absence of the protease is not known. In this study, we have characterized a protease-deficient mutant of HIV-1 in detail to determine its critically defective step in the viral replication phase. We demonstrate here that cleavage of Gag precursor is essential for the early viral replication phase, before and during a process of uncoating/reverse transcription.

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2. Materials and methods

2.1. Cell culture and DNA transfection

Human CD4+ leukemia cell lines, A3.01 [6], Molt4 clone 8 (M4-8) [7], and H9 [8], were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. A human colon carcinoma cell line, SW480 [9], was maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum. For transfection, uncleaved plasmid DNA was introduced into SW480 and leukemia cell lines by the calcium phosphate coprecipitation [9] and modified DEAE-dextran [10] methods, respectively.

2.2. Western immunoblotting

Lysates of transfected SW480 cells were processed for Western blotting as previously described using a serum from an HIV-1-infected individual [11]. [^{125}I]Protein A was used to detect viral Gag proteins.

2.3. Electron microscopy (EM)

Transfected SW480 cells were processed for EM as previously described [5].

2.4. RNA dot blot hybridization

Virion RNA was detected by the method as previously described [12]. Briefly, the same volume of culture supernatants obtained from SW480 cells transfected with wild-type (wt) or mutant clones was centrifuged to pellet virions, RNA was extracted from them, and RNA dot blot hybridization was performed using a full-length pNL432 genome as a probe. Some samples were treated with sodium hydroxide (4 N, 60°C for 15 min).

2.5. CAT and RT assays

The chloramphenicol acetyltransferase (CAT) [13] and reverse transcriptase (RT) [11] assays have been previously described.

2.6. Determination of Gag p24 antigen level

HIV-1 p24 antigen was detected by using a commercial ELISA kit (Abbot Laboratories).

2.7. Single-round replication assay

The process of HIV-1 replication was quantitatively analyzed by a system designated single-round replication assay (SRA) [14,15].

2.8. Polymerase chain reaction (PCR) analysis of viral DNA

Virus samples prepared from transfected SW480 cells were treated with DNAse I before infection to CD4+ cells to remove potentially contaminated plasmid DNAs. Samples for PCR amplification were prepared as described previously [14]. The detailed methods of PCR, agarose gel electrophoresis, and Southern blot hybridization were also as described previously [14].

2.9. DNA constructs

An infectious molecular clone of HIV-1 designated pNL432 and its protease-defective mutant pNL-Hc have been previously described [5]. An Env(−) mutant pNL-Nh was constructed from pNL432 as previously described [14,15]. Plasmids for SRA designated pNLnCAT (wt) and pNLnCAT-Nh (*env* mutant) were also described previously [14,15]. A mutation was introduced into the protease-coding region of pNLnCAT and designated pNLnCAT-Hc as previously described [5].

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3. Results

3.1. Phenotypic characterization of the protease-deficient mutant

A plasmid DNA designated pNL-Hc, which carried a mutation in the protease-coding region [5], was transfected into SW480 cells [9], and monitored for Gag expression in cells and RT production in culture supernatants. As shown in Fig. 1A, only the precursor of HIV-1 Gag was detected in cells transfected with pNL-Hc. In sharp contrast, mature *gag* gene products were readily seen in cells transfected with wt pNL432. Virion production by pNL-Hc into culture fluids as monitored by RT assay was relatively low, reaching only 10–20% of that by pNL432 (Fig. 1B). Essentially the same results were obtained by the enzyme-linked immunosorbent (ELISA) assay of Gag antigen (data not shown).

Although these data are consistent with those previously reported [3,5], we were still concerned about the level of virion production. Since only the precursor is produced in cells, measuring amounts of virions by RT assay and ELISA could result in an underestimation. We therefore monitored the virions produced from transfected SW480 cells by EM and hybridization. As shown in Fig. 2A, when transfected cells were examined by EM, while morphological abnormality of the mutant virions (doughnut-shaped) was easily seen, similar numbers of virions were observed. More quantitative analysis by RNA hybridization was performed to substantiate the EM observation. The same amount of culture supernatant was taken from SW480 cells transfected with pNL432 or pNL-Hc, subjected to ultracentrifugation, and RNA in the virions obtained was detected by hybridization as previously reported [12]. As is clear from Fig. 2B, no significant difference in RNA contents was detected.

Due to the effect of the mutation in the protease region described above, the NL-Hc virus lacked infectivity for A3.01, M4-8, and H9 cells (data not shown).

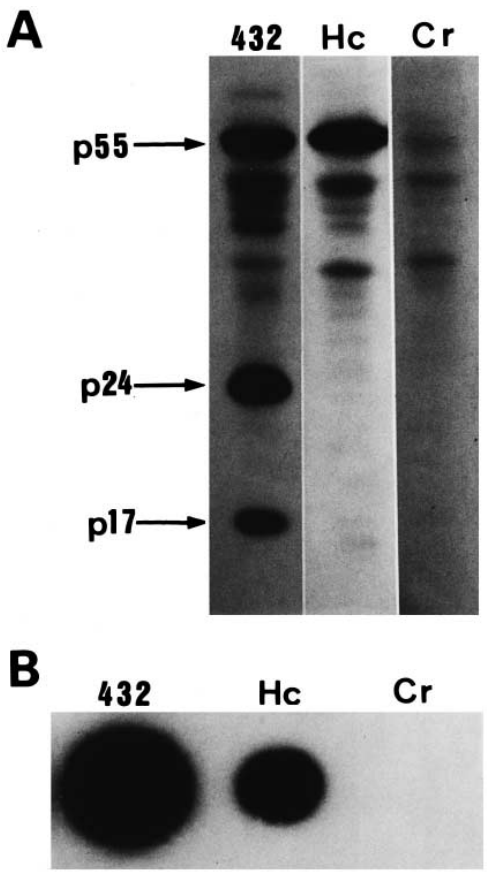

Fig. 1. Expression of viral proteins of the protease-deficient mutant. SW480 cells were transfected with 20 μg of pNL432 (432, wt), pNL-Hc (Hc, protease-deficient) or pUC19 (Cr, negative control), and monitored for the expression of Gag proteins (precursor p55; mature products p24 and p17) by Western blotting (A) [11] and for RT production (B) [11].

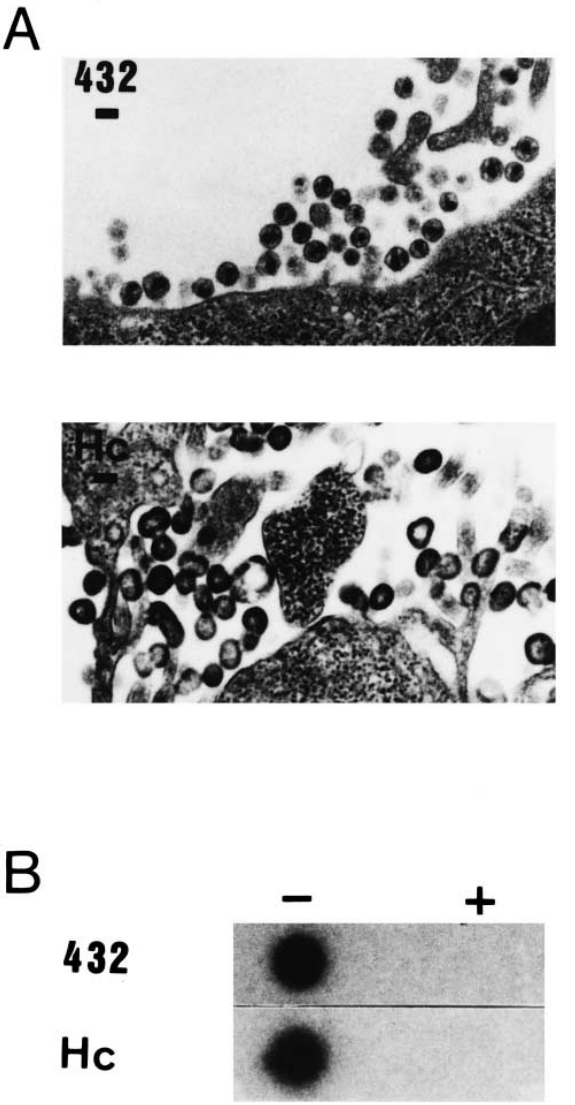

Fig. 2. Virion production of the protease-deficient mutant. SW480 cells were transfected with pNL432 (432, wt) or pNL-Hc (Hc, protease-deficient), and monitored for production of progeny virions by EM (A) [5] and by RNA dot blot without (−) and with (+) sodium hydroxide treatment (B) [12]. A: Bar = 100 nm.

3.2. Defective stage of the protease mutant in the viral replication cycle

The pNL-Hc was analyzed for its critical defect in the viral replication cycle. For this purpose, a system recently developed by us and designated SRA [14,15] was used. A proviral

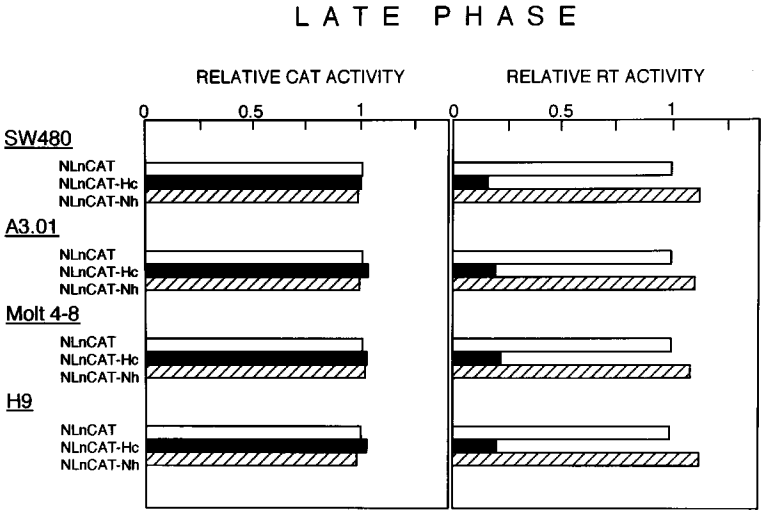

Fig. 3. Analysis of late viral replication phase by SRA. Four cell lines were co-transfected with one of three proviral CAT constructs plus prev1 (Rev expression vector), and 48 h later, CAT activity in cells and virion-associated RT activity in the culture supernatants were determined [14,15]. Relative activity is shown. CAT production is indicative of a normal proceeding of the transcription and translation in the late viral replication phase [14,15]. DNA constructs: pNLnCAT, wt; pNLnCAT-Hc, protease-deficient; pNLnCAT-Nh, Env(−).

clone carrying CAT was newly constructed and designated pNLnCAT-Hc. Because transfection of a viral DNA clone would bypass the early phase of viral replication (adsorption, penetration, uncoating/reverse transcription, integration), the results in Fig. 2 are consistent with the critical requirement of Gag cleavage in the early viral replication stage.

Fig. 3 shows the results obtained in four cell lines by the SRA for the late phase. Three CAT-proviral clones were introduced into the cell lines, and CAT and RT activities were determined. Although RT production in the culture supernatants by pNL-Hc was relatively low as shown in Fig. 1B, CAT activity in cells was normal. There was no difference in results among the four cell lines. The early phase of viral replication was examined by using CAT viruses prepared from SW480 cells transfected with the three proviral clones. The viruses were inoculated into the three cell lines, and CAT production was monitored. As demonstrated in Fig. 4, no significant CAT activity was expressed in cells infected with the NLnCAT-Hc virus relative to that in cells infected with the NLnCAT-Nh, a negative control Env(−) virus. There was, again, no cell difference.

The defect of the pNL-Hc was further analyzed by monitoring viral DNA synthesis (Fig. 5). Virus samples prepared from transfected SW480 cells were inoculated into A3.01 cells, and at 10 h post-infection, viral DNA was extracted, and Southern blot hybridization was performed [14]. As easily observed, while viral DNA was seen in cells infected with wt NL432, no specific DNA was detected in cells infected with the NL-Hc or the NL-Nh, a negative control Env(−) mutant virus. These results were reproduced in several independent experiments.

4. Discussion

In this study, we confirmed that, while a protease-defective mutant of HIV-1 is unable to process the Gag polyprotein precursor, morphologically abnormal non-infectious virions are produced from transfected cells (Figs. 1 and 2). Also in agreement with previous observations, cells transfected with the mutant generated much less RT activity than did cells transfected with wt clone (Figs. 1 and 3).

The SRA demonstrated that the transcription and translation process of the protease-defective mutant was normal (Fig. 3). Although a relatively low level of progeny virions was produced when monitored by RT assay (Fig. 1BFig. 3) and ELISA, a considerable number of doughnut-shaped virion-like particles were released from cells transfected with the protease-defective mutant clone (Fig. 2A). In addition, culture supernatants from the mutant-transfected cells contained a normal level of particle-associated viral RNA (Fig. 2B). It is quite likely, therefore, that the unprocessed Gag-Pol fusion protein had lower specific RT activity and weaker immunoreactivity.

A clear defect in the early viral replication phase was de-

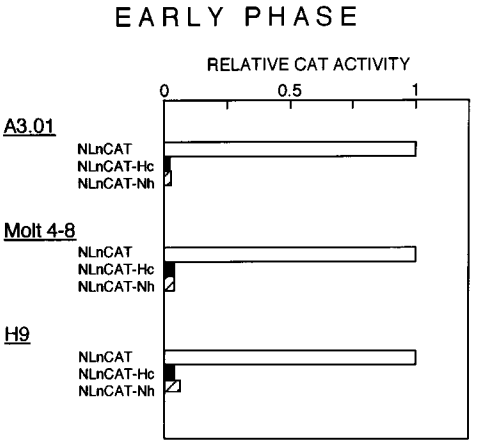

Fig. 4. Analysis of early viral replication phase by SRA. Virus samples (5×10^5 RT units) obtained from co-transfections of pNLnCAT constructs and prev1 into SW480 cells (Fig. 3) were inoculated into CD4+ cells (2×10^6) indicated, and 48 h later, CAT activity in cells was determined [14,15]. Relative activity is shown. Viruses: NLnCAT, wt; NLnCAT-Hc, protease-deficient; NLnCAT-Nh, Env(−). Essentially the same results were obtained when input viral dose was normalized by Gag Elisa.

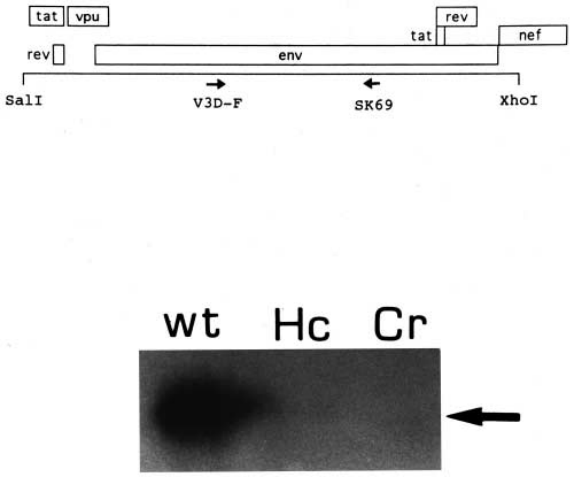

Fig. 5. PCR analysis of viral DNA. Total DNAs prepared from A3.01 cells infected with NL432 (wt), protease-deficient NL-Hc (Hc), or Env(−) NL-Nh (Cr) were subjected to PCR amplification and a specific product was detected by Southern blot hybridization [14]. Location of the primers V3D-F and SK69 [14] is indicated. As a probe, ^{32}P-labelled *Sal*I-*Xho*I fragment was used. The arrow represents the size (937 bp) of the amplified product predicted from the location of the primers.

tected by our SRA (Fig. 4). In fact, no viral DNA was found in cells infected with the protease-defective mutant (Fig. 5). This result showed that there is a critical step(s) for the replication of the mutant before or during a process of uncoating/reverse transcription. A role of the protease in the early phase was also suggested by a study using protease inhibitors [16]. It is unlikely that the entry step into cells mediated by Env proteins of the mutant is defective [17]. This needs, however, to be experimentally demonstrated.

Our results presented here strongly suggest that cleavage of Gag precursor does not affect the release of virions and that virions produced in the absence of functional viral protease are most likely to be defective at the process of uncoating/reverse transcription. It should be noted that the defect of many in-frame *gag* mutants appears to be located at this step (our unpublished observation).

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