Enhancement of human immunodeficiency virus type 1 infectivity by Nef is producer cell-dependent

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The growth kinetics of wild-type and nef mutant viruses of human immunodeficiency virus type 1 were comparatively analysed in several human CD4+ cell lines. Delayed replication of nef mutant virus was observed in all cell lines examined. To determine the stage in the virus replication cycle that is affected by Nef, a single-round replication assay was performed. Initially, the expression of marker genes in transfected cells was examined in order to study the role of Nef in the late phase of infection. The results obtained indicated that Nef is dispensable during the transcription to virion production stage. Next, the effect of Nef on the early phase was investigated with a single-round infection. It was demonstrated that Nef is required in the early phase of the virus replication cycle, from virion adsorption to integration. Finally, the infectivity of virus stocks prepared from four cell lines was determined. The relative infectivity of the nef mutant from the four cell lines differed. Taken together, we conclude that Nef acts via modulation of viral particles to enhance virus infectivity in a cell-dependent manner.

Introduction

One of the accessory genes of human immunodeficiency virus type 1 (HIV-1), designated nef, encodes a 25 or 27 kDa protein. In early studies, it was suggested that the gene product, Nef, acted as a negative factor in virus replication (Terwilliger et al., 1986) by repressing transcription from the long terminal repeat (LTR) promoter (Ahmad & Venkatesan, 1988; Niederman et al., 1989). However, these findings were not confirmed in a subsequent report (Hammes et al., 1989). Several studies have reported that Nef could moderately facilitate the virus replication rate (de Ronde et al., 1992; Kim et al., 1989; Terwilliger et al., 1991; Zazopoulos & Haseltine, 1992, 1993). However, a drastic effect of Nef as a positive factor has been observed in experimental infections in vivo, which demonstrated that Nef of simian immunodeficiency virus SIVmac is necessary for maintaining high virus loads and for disease progression of AIDS in rhesus monkeys (Kestler et al., 1991). This result was supported by Jamieson et al. (1994) using HIV-1-infected severe combined immunodeficient mice which had been transplanted with foetal human thymus and liver tissues.

Subsequently, this positive effect was clearly revealed in in vitro infection experiments using quiescent primary T-lymphocytes (Miller et al., 1994; Spina et al., 1994) and was further observed in CD4+ cell lines infected with low virus inputs (Chowers et al., 1994). In the latter observation, it was also suggested that Nef enhances viral particle infectivity (Chowers et al., 1994). Miller et al. (1995) have recently shown that the increased infectivity by Nef is independent of virus entry, and is manifested at the stage after entry but prior to or coincident with viral gene expression. Schwartz et al. (1995) have indicated that Nef acts at an early stage of the virus replication cycle, but not when the virus binds to the receptor and before the completion of reverse transcription. Moreover, Aiken & Trono (1995) have suggested that Nef functions at the stage of particle formation, leading to the efficient completion of proviral DNA synthesis, not to enhanced virus internalization. This study was confirmed by Chowers et al. (1995).

In the present study, we firstly confirmed the delayed replication kinetics of nef mutant virus in several cell lines. To determine the replication stage influenced by Nef, we used the single-round replication assay (Helseth et al., 1989; Sakai et al., 1995). The results obtained showed that Nef is dispensable in
the stage from transcription to virion production but is required in the early phase of the virus replication cycle, from virion adsorption to integration. These findings are in agreement with previous reports (Aiken & Trono, 1995; Chowers et al., 1995; Miller et al., 1995; Schwartz et al., 1995). We finally examined the infectivity of the nef mutant virus produced in several cell lines. The relative infectivity of the mutant differed depending on the cells in which virus was produced.

Methods

**Cell culture and transfection.** A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated foetal calf serum (FCS). CD4+ human leukaemia cell lines H9 (Popovic et al., 1988), CEMx174 (Salter et al., 1985), A3.01 (Folks et al., 1985), and M8166 (Shibata et al., 1991) were maintained in RPMI 1640 medium containing 10% FCS.

For transfection, uncleaved plasmid DNA was introduced into SW480 cells by the calcium phosphate co-precipitation method (Graham & Van der Eb, 1973; Wigler et al., 1979) and into H9, CEMx174, M8166 and A3.01 cells by the modified DEAE-dextran method (Takai & Ohmori, 1990).

**Infection.** Culture supernatant was harvested, filtered through a 0.45 µm pore filter (Millipore), and assayed for reverse transcriptase (RT) activity. The virus stocks were stored at −80 °C. H9, CEMx174, A3.01 and M8166 cells were infected by incubating 10⁶ cells with virus supernatants, adjusted for identical RT activity or p24 concentration (determined by HIV-1 p24 antigen ELISA; Cellular Products), in the presence of 2 µg polybrene.

**RT assay.** Virion-associated RT activity was measured as described previously (Willey et al., 1988). For quantification, spots on DE81 paper were cut out and RT activity was determined by scintillation counting.

**CAT assay.** The chloramphenicol acetyltransferase (CAT) assay has been previously described (Gorman et al., 1982). CAT levels were assayed in equivalent amounts of cell lysates from transfected SW480 or CEMx174 cells, and from infected H9, CEMx174 or M8166 cells.

**DNA constructs.** An infectious proviral clone of HIV-1, pNL432 [pNL-wild-type (wt) and its mutants, designated pNL-Xh (nef mutant) and pNL-Ss (vpu mutant)], have been described previously (Adachi et al., 1986, 1991). pNL-SsXh (vpu-nef double mutant) was constructed from pNL-Ss by digestion with restriction enzyme XhoI, blunt-ending with T4 DNA polymerase, and resealing with T4 DNA ligase. To make pNLenCAT, the CAT coding sequence of pSV2CAT (784 bp HindIII–SmaI fragment) (Sakai et al., 1990) was inserted into the SpI site (at nucleotide 6153) in the vpu gene and the DraI site (at nucleotide 6591) in the env gene of pNL432. pNLenCAT-Xh was constructed from pNLenCAT by digestion with XhoI, blunt-ending with T4 DNA polymerase, and resealing with T4 DNA ligase. An nru expression vector pNLnABS was generated from pNLJABS (Shibata et al., 1995) by introducing the mutation into the XhoI site as described above, and pNLnABS-Nh, used as an nru negative control, was generated from pNLnABS by blunt-end ligation of the NheI site.

Results

**Growth kinetics of nef mutant virus in various CD4 cell lines**

In preliminary experiments, the growth ability of wt and nef mutant viruses prepared from SW480 cells transfected with pNL-wt or pNL-Xh was examined in H9, CEMx174, A3.01 and M8166 cell lines. Although the effect of the nef mutation was small, the nef mutant virus always showed delayed growth kinetics relative to those of wt virus (data not shown), confirming reports that Nef is a positive factor in virus replication (Chowers et al., 1994; Kim et al., 1989; Terwilliger et al., 1991; Zazopoulos & Haseltine, 1992, 1993). To substantiate the minor difference in growth kinetics between wt and nef mutant viruses, we introduced an additional vpu mutation, which abrogates efficient virion production (Gott-
Function of HIV-1 Nef

Fig. 2. Single-round replication assay. (a) Env-deficient pNLenCAT and env expression vector pNLnΔBS constructs used for a single-round replication assay. (b) Schematic representation of a single-round replication assay. CAT and RT production in transfected cells is indicative of a normal late replication phase (from transcription to virion release). CAT production in cells infected with NLenCAT virus is indicative of the progression of the early phase of the virus replication cycle (from attachment to integration).

Fig. 3. Marker gene expressions in two cell lines transfected with pNLenCAT-wt plus pNLnΔBS-wt or with pNLenCAT-Xh (nef mutant) plus pNLnΔBS-wt. SW480 and CEMx174 cells were co-transfected with 20 µg pNLenCAT-wt or pNLenCAT-Xh and 10 µg pNLnΔBS-wt; 48 h later, CAT activity in the lysates prepared from transfected cells and RT activity in the culture supernatants were determined.

linger et al., 1993; Klimkait et al. 1990; Sakai et al., 1995), into the nef mutant and monitored the replication of the nef-vpu and vpu mutant viruses. The four CD4+ cell lines were infected with the mutant viruses obtained from SW480 cells transfected with pNL-Ss or pNL-SsXh. As clearly observed in Fig. 1, the nef-vpu double mutant grew much less than the vpu mutant in all cell lines examined. These results provide strong evidence that Nef plays a positive role in virus replication.
K. Tokunaga and others

**Fig. 4.** Virion production in various cell lines transfected with pNL-wt or pNL-Xh (nef mutant). H9, CEMx174, A3.01 and M8166 cells were transfected with 20 µg pNL-wt or pNL-Xh; 48 h later, RT activity in the culture supernatants was determined.

**Determination of the virus replication stage affected by Nef**

To determine the stage in the virus replication cycle when Nef is required, we performed a single-round infection assay, which is similar to that described previously (Helseth et al., 1989; Sakai et al., 1995). To do this, env-vpu-deficient proviral clones carrying a marker CAT gene with or without intact nef (pNLenCAT and pNLenCAT-Xh, respectively), an env expression vector without nef (pNLnΔBS), and its env-deficient mutant (pNLnΔBS-Nh) were constructed (Fig. 2a). In this system, when Env is supplied from pNLnΔBS in trans, pNLenCAT is able to produce infectious particles carrying CAT and undergo one round of replication (Fig. 2b). Efficiencies of transcription/translation and virion production in the late virus replication phase can be determined by monitoring CAT activity in transfected cells and RT activity in supernatants from these cells, respectively. CAT activity in cells infected with the CAT-carrying virus represents the efficiency with which early replication phase (from virus entry to integration) proceeds.

As shown in Fig. 3, upon co-transfection into SW480 cells, pNLenCAT-Xh (nef mutant) and pNLnΔBS-wt expressed CAT activity similar to that of the wt clone. The amount of mutant progeny released into the culture medium, as monitored by RT activity, was also comparable to that of the wt clone. The same experiment was carried out in a lymphocytic cell line (CEMx174) with similar results (Fig. 3). Furthermore, in the other transfection experiments, pNL-wt and pNL-Xh, the parental clones, behaved exactly like pNLenCAT-wt and pNLenCAT-Xh in H9, CEMx174, A3.01 and M8166 cells (Fig. 4). These results indicated that Nef is dispensable for the stage from transcription to virion production in the late phase of the virus replication cycle.

To determine whether Nef is required in the early stage of virus replication, progeny CAT-carrying virions obtained from transfections (Fig. 2b) were inoculated into H9, CEMx174 and M8166 cells, and CAT activity was monitored. As shown in
Fig. 6. Infectivity of nef mutant virions produced in various cell lines. Infectivity of cell-free virus samples prepared from transfected cells indicated was assessed by infecting MAGI cells and counting blue foci 48 h later (Kimpton & Emerman, 1992). Each infection was performed in triplicate; the average infectivity and standard deviation is shown. Xh, nef mutant virus.

The infectivity of virions produced in various cell lines

We then examined how the early defect in the nef mutant virions is reflected in infectivity. Virions were prepared from various cells transfected with wt or nef mutant clones, and their infectivity was determined by a single-cycle infection assay (MAGI assay; Kimpton & Emerman, 1992). As shown in Fig. 6, the infectivity was expressed as the number of infected cells per 20–100 pg p24 antigen. Although the nef mutant virus was always less infectious, the magnitude of the defect varied. In these experiments, a 3- to 5-fold greater infectivity was consistently observed for wt viruses prepared from M8166, H9 and A3.01 cells. Of note, an approximately 11-fold reduction in infectivity was observed for the nef mutant virus produced in CEMx174 cells.

Discussion

In recent studies, Nef has been reported to be a positive rather than a negative factor in virus replication (Chowers et al., 1994; de Ronde et al., 1992; Kim et al., 1989; Miller et al., 1994; Spina et al., 1994; Terwilliger et al., 1991; Zazopoulos & Haseltine, 1992, 1993). In this regard, our data in the present work are perfectly consistent with these reports. Our results show that the growth of nef mutant virus was clearly delayed relative to that of parental virus (Fig. 1).

We detected no difference in the levels of CAT and RT production of wt and nef mutant viruses in the several cell lines transfected with each proviral DNA clone (Figs 3 and 4). These results indicate that Nef plays no obvious role at the late stage of virus replication. It was also demonstrated that Nef plays an important role in the early phase of replication, from adsorption to integration, as indicated by CAT production in cells infected with wt or nef mutant virus carrying the CAT gene (Fig. 5). This result is in agreement with previous reports (Aiken & Trono, 1995; Chowers et al., 1995; Miller et al., 1995; Schwartz et al., 1995), in terms of the replication phase affected by Nef.

In the present work, the infectivity of the nef mutant prepared from several producer cell lines was also monitored. Considerable differences in the relative infectivity of the mutant were noted in the different cell lines (Fig. 6).

The conclusion of this report that Nef acts at the very late stage of virus replication, such as assembly, release, and maturation, to enhance the efficiency of the early replication process in a producer cell-dependent way raises the obvious possibility that Nef may interact with the virion structural proteins. Thus far, we have detected no significant difference in virion proteins between wt and nef mutant virus as judged by immunoprecipitation (our unpublished results), which is in agreement with the recent report by Schwartz et al. (1995). A more detailed analysis of structural components of virions, produced in the presence and the absence of Nef, in various cell lines, will allow a better understanding of the mechanism by which Nef influences virus infectivity.

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References


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