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Substitution of HIV Type 1 Nef with HTLV-1 p12

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Abstract

Human retroviruses, such as HTLV-1 and HIV-1, encode accessory proteins, which regulate viral pathogenesis. The p12 protein of HTLV-1 is encoded from the pX-I open reading frame, and is critical for efficient virus replication in rabbits. Although dispensable for infection, replication, and immortalization of activated lymphocytes in culture, p12 expression is important for infection of quiescent lymphocytes. Similar to HTLV-1 p12, Nef is important for virus infectivity in SIV animal models. We questioned whether p12 could replace Nef in HIV-1, and reconstitute virus replication in culture. We found that p12 could complement for effects of Nef on HIV-1 infection of Magi-CCR5 cells or macrophages.

INTRODUCTION

Human T cell leukemia type 1 (HTLV-1) infects and immortalizes human CD4 T cells *in vitro* and is associated with the development of adult T cell leukemia/lymphoma (ATLL).^{1,2} HTLV-1 is a complex retrovirus. The 3' region of its genome, called pX, encodes the unique viral accessory proteins, in addition to the structural genes common to most retroviruses (i.e., *gag*, *pol*, and *env*). The pX region contains four open reading frames, termed pX-I to pX-IV. pX-III and pX-IV encode the posttranscriptional regulator protein Rex and the viral oncoprotein, Tax, respectively, which are well characterized.³ On the other hand, much less is known regarding the roles of HTLV-1 replication and pathogenesis of pX-I- and pX-II-encoded proteins p12, p13, and p30.

The pX-I-encoded protein p12 consists of 99 amino acids (aa) and is highly conserved in related viruses HTLV-1, 2, and simian T cell leukemia virus type 1.⁴ The protein has four proline-rich SH-3 binding motifs (PXXP), which are found in proteins involved in the intracellular signaling pathway (Fig. 1). Recently, several groups demonstrated that expression of p12 induces nuclear factor of activation of T cells (NF-AT) and transcriptional factor Stat 5 activation in T cells suggesting that p12 may alter T-cell signaling.^{5–7} Using a p12-deficient mutant within an HTLV-1 molecular clone ACH, we demonstrated that p12 is dispensable for replication in B5 macaque cells (T. Tsukahara and L. Ratner, unpublished observations), and immortalization of human peripheral blood lymphocytes (PBLs) in the presence of interleukin-2 (IL-2).⁸ However, we and others showed that p12 is important for viral infectivity in quiescent PBL and the establishment of persistent infection in rabbits.^{9,10}

Human immunodeficiency virus type 1 (HIV-1) encodes a Nef protein. It consists of 206 aa, and it is highly conserved in HIV-1, 2, and simian immunodeficiency virus (SIV).¹² Similar to HTLV-1 p12, Nef has a PXXP motif.¹² Nef modulates T cell signaling including T cell

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receptor activation and expression of NF-AT, and it interacts with cellular tyrosine kinases Hck and Fyn through PXXP motifs.^{13–15} Although Nef is dispensable for infectivity in culture, it is important for efficient infectivity of quiescent PBLs *in vitro*, and in SIV-infected animal models.^{16,17} Thus, HTLV-1 p12 and HIV Nef are structurally distinct retroviral accessory proteins, which are able to alter some T cell signaling pathways and play a critical role in enhancing viral infectivity in primary lymphocytes and infected animals.

MATERIALS AND METHODS

To construct Nef mutants, p102.ATG or p125.ATG clones, a six-nucleotide sequence including the initiation codon of Nef, GATGGG was replaced with CTCGAG (*Xho*I site) by a polymerase chain reaction, and then the viral clones were digested with *Xho*I and religated, to remove the region of Nef encoding 1–33 aa (Fig. 2).¹⁸ For chimeric p102.p12 or p125.p12 clones a full-length p12 cDNA from an HTLV-1 molecular clone ACH was inserted into the Nef coding region at the *Xho*I site of p102.ATG or p125.ATG (Fig. 2). The primers used for p12 were as follows: a forward primer, 5'-ccgctcgaggcactatgctgttcgcttcag-3', and reverse primer 5'-ccgctcgagcttagaagaggaaagccgcg-3', respectively. The frame shift Nef mutants p102.Xh or p125.Xh clones were used as described previously.^{18,19}

Two micrograms of each proviral clone was transfected by a calcium precipitation method, and viral proteins were analyzed by immunoblot using cell lysates obtained 48 hr posttransfection.^{20,21} Cell lysates were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblots were performed using an HIV-1 patient serum, followed by treatment with an antihuman horseradish peroxidase-conjugated secondary antibody and visualization was with an ECL western detection system (Amersham, United Kingdom).

To check the presence of virus particles, the p24^{gag} in the supernatants from the 293T transfected cells was quantified by a p24 antigen enzyme-linked immunosorbent assay (p24 ELISA) (Coulter Corp., Hialeah, FL).

Expression of p12 mRNA was examined by an RNase protection assay (RPAIII kit, Ambion, Austin, TX), since there are no antibodies against p12. Total RNA was isolated from 293T cells transfected with each HIV-1 clone at 48 hr posttransfection, and hybridized with ³²P-labeled p12 antisense RNA probe (263 nt). The protected RNAs (188 nt) were separated on a 5% acrylamide gel with 8 M urea and visualized by autoradiography.²¹ The mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in all samples were analyzed as an internal control (Pharmingen, San Diego, CA).

For quantitation of infectious particles, we used Magi-CCR5 cells, which express CD4, CXCR4, and CCR5 on the surface, and contain a reporter gene β -galactosidase (β -Gal) driven by the HIV-1 long terminal repeat.²² The indicator cell line allowed detection of X4 or R5 HIV-1 strains by β -Gal staining after a single cycle of replication. Virus stocks were generated by transfection of 293T with each wild-type, Nef mutant, or chimeric HIV-1.p12 clones at 48 hr posttransfection, and examined for p24^{gag} concentration by the p24 antigen ELISA. Inoculation of Magi-CCR5 cells (4×10^4) was initiated with various amounts of filtered virus supernatants. At 48 hr after infection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside as described previously, and the number of blue cells enumerated.²² Under these conditions the dilution of the viral stocks and the number of infected cells were in the linear range.

PBLs were purified by Ficoll–Hypaque density gradient centrifugation, and were obtained from healthy donors and stimulated with 5 μ g/ml phytohemagglutinin and 50 units IL-2 for 3 days. Inoculation of PBLs (1×10^6) was initiated with 100 or 20 ng of p24^{gag} in the presence

of IL-2. Supernatants from these cultures were collected every 3 or 4 days and stored. Fresh PBLs were added weekly as feeder cells. The infectivity of clones was monitored by a reverse transcriptase (RT) assay, as described previously.²³

Terminally differentiated, noncycling macrophages were infected with equal amounts of p24^{gag} from a wild-type macrophage tropic p125 clone, Nef mutant, and chimeric p12 clones generated in 293T cells transfected with proviral clones. Infections were initiated with 100 or 4 ng of p24^{gag}.

RESULTS

In this study, we examined whether p12 could replace effects of Nef in viral replication. Therefore, Nef-defective mutants and chimeric HIV-1 clones containing p12 were generated within the T cell line-tropic NL4-3 (p102) or macrophage-tropic (p125) strain, which is a chimera of the NL4-3, HXB2, and ADA strains of HIV-1 (Fig. 2).²³

To confirm that chimeric HIV-1.p12 clones were able to produce Gag and Env proteins, wild-type p102 or p125 clone, Nef mutant, and chimeric HIV-1.p12 clones were transfected into 293T cells, and viral proteins were analyzed by immunoblot using cell lysates obtained 48 hr posttransfection. Immunoblot analysis with the patient serum showed similar levels of Gag proteins including p55^{prgag}, p24^{CA}, p17^{MA} and p41, as well as envelope proteins gp160 and gp120 for each HIV-1 clone (Fig. 3A). Envelope expression was faint but with prolonged exposure, bands were readily detectable. All clones tested produced similar amounts of p24 antigen (data not shown). To confirm that Nef mutants were unable to produce Nef proteins, cell lysates were used as described above. Immunoblot analysis with a polyclonal Nef antibody revealed that Nef protein was expressed from clones with a wild-type Nef coding sequence, but not from those with mutations in the Nef coding domain (Fig. 3B). Chimeric p102.p12 and p125.p12 clones showed similar levels of p12 mRNA compared to that of a p12 expression vector driven by mouse stem cell virus promoter (MSCV-p12) as a positive control (Fig. 3C). No p12 mRNA was observed in total RNAs from cells transfected with a wild-type p102 clone or untransfected 293T cells.

We then examined whether p12 could substitute for the effects of Nef on HIV-1 replication. Over 500 infected cells were observed in a well with a wild-type T cell line-tropic p102 clone at the highest virus concentration (Fig. 2). Nef-defective mutants p102.Xh and p102.ATG showed approximately 50% reduction in infectivity at each virus concentration, compared to the wild-type p102 strain. This observation was consistent with the data that Nef enhances infectivity of HIV-1 in these cells.²⁴ The infectivity of chimeric p102.p12 clones was also reduced similar to Nef mutants. On the other hand, over 200 infected cells were detected with a wild-type macrophage-tropic p125 clone. Nef mutants p125.Xh and p125.ATG also showed approximately 50% of wild-type infectivity. Interestingly, the infectivity of chimeric p125.p12 was comparable to the wild-type p125 clone in each case. These findings suggest that p12 may complement effects of Nef on infection of the HIV-1 p125 clone in Magi-CCR5 cells.

Finally, we examined effects of p12 on macrophage infection. At high viral inoculation, Nef mutant virus was released two- or three-fold less efficiently than wild-type virus, as expected (Fig. 4).²⁵ Chimeric p12 virus was released two- or threefold even more efficiently than the wild-type virus. Similar results were observed after inoculation of macrophages with lower titers of virus. These findings suggest that p12 may substitute for effects of Nef on infection of the p125 clone in macrophages. However, p12 did not compensate for loss of Nef in replication studies in proliferating PBLs.

DISCUSSION

In this study, we questioned whether p12 could substitute for effects of Nef on viral replication in culture systems through effects at the same or a different virus replication step modulated by Nef. We showed that p12 rescued effects of Nef on HIV-1 infection of Magi-CCR5 cells or macrophages using a macrophage-tropic strain of HIV-1. These findings suggest that Nef and p12 may represent examples of convergent evolution for efficient retrovirus infection.

We constructed chimeric HIV-1 clones that contain p12 in places of Nef (Fig. 2). The resulting viral clones highly expressed p12 mRNA, which was used as a surrogate for the presence of p12 protein. Nef protein was not detected in 293T cells transfected with these clones. Immunoblot analysis of Gag and Env and the p24 antigen ELISA demonstrated that there were no differences in viral protein expression between the wild-type and chimeric p12 clones, suggesting that HTLV-1 p12 did not affect viral transcription or translation (Fig. 3).

Expression of p12 in a macrophage-trophic HIV-1 strain complemented for effects of Nef on viral infectivity of Magi-CCR5 (Fig. 2), suggesting that p12 may be important for the initial step of infection since this is a single round replication assay. However, this p12 function was not found in a T cell line-tropic virus, although Nef enhances viral infectivity independently of HIV-1 tropism.²⁶ We do not know the reason for the discrepancy. Using a macrophage-tropic HIV-1 clone, p12 complemented the effects of Nef on viral infectivity on macrophages suggesting that p12 may be important for efficient infection in quiescent and nondividing cells (Fig. 4).

The p12 protein is a positive factor for HTLV-1 infectivity in quiescent cells.⁹ However, its mechanism of action is not clear. Although the mechanism by which Nef enhances viral infectivity is also not fully understood, there are several possible explanations. Nef does not modify the ability of the virus to fuse or bind to target cells, suggesting that Nef may function at a postentry step in the viral life cycle.²⁷ Indeed, virions produced in Nef-positive cells were shown to more efficiently initiate reverse transcription.^{28–30} Thus, p12 may also affect viral DNA synthesis.

Nef also modifies activation of infected cells in culture.³¹ Expression of Nef in a T cell line, Jurkat, induces IL-2 in the presence of CD3 and CD28 antibody, but cell growth is not changed.^{31,32} Therefore, we established stable p12-expressing Jurkat cells (Jurkat-p12) using a retroviral vector system (data not shown). In contrast to HIV-1 Nef, IL-2 induction was not observed in Jurkat-p12 cells under the same experimental conditions, as determined by an IL-2 ELISA (Ebioscience, San Diego, CA). However, the clones of Jurkat cells expressing the highest levels of p12 exhibited a more rapid rate of cell proliferation than the parental cells, especially under low serum conditions (data not shown). Cell cycle analysis showed accelerated transition of G₁ cells to S phase in the Jurkat-p12 cells than in the parental cells, under these conditions. Thus, p12 may be directly or indirectly involved in cell cycle regulation. If so, this might provide an explanation for p12 effects on viral infectivity in quiescent cells. Interestingly, apoptosis was also reduced in Jurkat-p12 cells with serum starvation compared to the parental cells (data not shown). Nef also prevents cells from undergoing apoptosis through activation of the Fas receptor, and interaction between Nef and an apoptosis regulator ASK1 is associated with this Nef function.^{33,34} Therefore, p12 may be involved in resistance of apoptosis observed in HTLV-1-infected cells.^{3,35} However, additional studies of p12 mutants are required to determine the relevance of these findings to the described effects on HIV-1 replication.

The p12 protein has particular protein motifs including four PXXP motifs, two leucine zipper motifs, a dileucine motif, and IL-2 receptors binding site (Fig. 1).⁴ In contrast, Nef has a PXXP motif and a CD4 binding site. Mutations in the PXXP sites of p12 did not alter HTLV-1

infectivity or NFAT activation (data not shown). Moreover, p12 localizes to the endoplasmic reticulum and *cis* Golgi apparatus, whereas Nef has been found in membrane, cytoskeletal, and cytosolic fractions.^{36,37} We do not know which domains could be responsible for effects of p12 on retroviral infection. Our chimeric HIV-1 clones containing the p12 open reading frame could be a useful tool for analyzing the role in viral infectivity of each domain of p12. We found that p12 substituted for Nef in our culture system. These findings suggest that Nef and p12 may represent examples of convergent evolution for efficient retrovirus infection, and will help to define p12-mediated effects on infectivity of HTLV-1.

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FIG. 1.

Diagram of p12 and Nef with predicted functional motifs. aa, amino acid; TM, transmembrane region; LZip, leucine zipper motif; DxxLL, dileucine motif; PxxP, SH-3 binding motif; MGG, myristoylation site; EEEE, required for MHC I down-regulation; RR, NAK binding site; LL, AP-2 binding site.

FIG. 2.

Structure of HIV.p12 chimeric viruses. The panel shows mutants in a T cell line-tropic strain of HIV-1, NL4-3, also designated p102, and a macrophage-tropic strain, NL4-3.ADA, also designated p125. In each case a frameshift mutation was introduced at the *XhoI* (Xh) at residue 33 of the Nef coding sequence, a deletion of the first 33 codons of Nef, or substitution of the p12 coding sequence, including a termination codon (*). MAGI-CCR5 cell infectivity assays with HIV.p12 chimeric viruses. The number of blue cells was determined on plates after infection with 75, 15, or 3 ng of NL4-3-based viruses, or 225, 150, or 75 ng of NL4-3.ADA-based viruses. Similar results were obtained in three independent experiments.

FIG. 3.

Gene expression from HIV.p12 chimeric viruses. Each of the chimeric or parental HIV clones was transfected into 293T cells, and cell lysates analyzed by immunoblot for **(A)** Gag and Env expression with HIV-infected patient anti-serum, or **(B)** Nef expression with rabbit antiserum, or **(C)** by RNase protection assay for p12 mRNA expression. Positions of molecular mass standards are indicated on the left side of **A** and **B**. Positions of input probe are indicated on the left side of **C**.

FIG. 4.

Replication of HIV.p12 chimeric viruses in primary macrophages. Macrophages were infected with 100 ng (left) or 4 ng (right) of each virus, and cell-free reverse transcriptase activity expressed as cpm/ml. Similar results were obtained at the indicated doses in three independent experiments with three different donors.