JOURNAL OF VIROLOGY, Sept. 1996, p. 6493–6496 Vol. 70, No. 9
0022-538X/96/$04.00+0

The Association of Nef with a Cellular Serine/Threonine Kinase and Its Enhancement of Infectivity Are Viral Isolate Dependent

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Received 13 May 1996/Accepted 14 June 1996

The *nef* genes of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) encode a 27- to 34-kDa myristoylated protein which induces downregulation of CD4 surface levels and enhances virus infectivity. In adult macaques, Nef has been implicated in pathogenesis and disease progression. Both HIV-1 SF2 Nef and SIVmac239 Nef have been shown to associate with a cellular serine/threonine kinase. We tested five functional Nef isolates to examine whether this kinase association is a property conserved among different isolates. HIV-1 SF2 and 248 and SIVmac239 Nef proteins were found associated with the kinase. HIV-1 NL4-3 and 233 Nef proteins were found weakly associated or not associated with the kinase. All five Nef isolates efficiently downregulated CD4 cell surface expression, suggesting that the association with this cellular kinase is not required for Nef to downregulate CD4. Comparison of the SF2 and NL4-3 isolates shows a differential ability of Nef to enhance infectivity that suggests a possible correlation between kinase association and enhancement of infectivity.

Nef is encoded by an open reading frame that overlaps the 3′ long terminal repeat in human immunodeficiency virus type 1 (HIV-1) and HIV-2 and simian immunodeficiency virus (SIV) (3, 6, 32). Nef, together with Tat and Rev, is expressed early during virus infection from several multiply spliced mRNAs (17, 20, 26, 28). Nef expression is essential for the maintenance of high levels of viral replication and pathogenesis in adult macaques infected with SIV (16). The significance of Nef in virus replication and pathogenesis has been highlighted by recent findings showing that HIV isolates from some long-term nonprogressors have deletions in the *nef* gene (9). A positive effect of Nef in virus replication and pathogenesis has also been observed in HIV-infected SCID-hu mice (2, 14). Nef has been found to enhance replication of HIV-1 in vitro in primary blood lymphocytes (25, 33). In addition to enhancing viral replication, Nef possesses another important function, i.e., to downregulate cell surface expression of CD4 (1, 4, 5, 7, 10, 11, 13, 24), the receptor for both HIV and SIV (8, 15, 19, 21, 23). Recent reports suggest that HIV and SIV Nef proteins are associated with a cellular serine/threonine kinase (29, 30). Interestingly, Nef itself does not appear to be phosphorylated by this kinase (29, 30). In this report, we show that association of Nef with the cellular serine/threonine kinase is Nef isolate dependent and suggest a possible correlation between kinase association and enhancement of infectivity.

Nef association with a cellular serine/threonine kinase is isolate dependent. HIV-1 SF2, NL4-3, 233, and 248 and SIVmac239 Nef proteins were expressed individually in HuT 78 cells as we have described before (4, 5, 22). All five Nef proteins were stably expressed at comparable levels (Fig. 1A) and were found to be functional as indicated by their ability to induce CD4 cell surface downregulation (Fig. 1B). NL4-3, 233, and 248 Nef proteins have a faster mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) than does SF2 Nef. Like the 233 and 248 Nef proteins, NL4-3 Nef has four fewer amino acids than does SF2 Nef and also has an alanine residue at position 54, and these factors together are responsible for the difference in mobility (5, 27). In vitro kinase assays were performed with immunoprecipitates from the HuT 78 cells expressing the different Nef isolates. As previously described, HIV-1 SF2 and SIVmac239 Nef proteins associated with the kinase (29, 30) (Fig. 2, lanes 2 and 7). The 248 primary isolate of Nef was also found to associate with the kinase (Fig. 2, lane 5). By contrast, HIV-1 NL4-3 and 233 Nef proteins were found to be weakly associated or not associated with the kinase (Fig. 2, lanes 3 and 4). These results suggest that the association of Nef with the cellular kinase is isolate dependent. In addition, the fact that all five isolates induced cell surface CD4 downregulation indicates that Nef's association with the serine/threonine kinase is not essential for this function.

Failure of NL4-3 Nef to associate with the serine/threonine kinase is cell type independent. To determine if the failure of NL4-3 Nef to associate with the kinase is cell type dependent, U937 cells were transduced to express either SF2 or NL4-3 Nef. SF2 Nef and NL4-3 Nef were expressed at similar levels as determined by Western blot (immunoblot) analysis (Fig. 3A), and both were functional as determined by their ability to suppress CD4 surface expression (data not shown). In vitro kinase assays performed with SF2 Nef immunoprecipitates clearly showed a phosphorylated band of approximately 62 kDa, whereas similar assays performed in parallel with NL4-3 Nef immunoprecipitates showed a barely detectable band (Fig. 3B). These results confirm, with a different cell type, the differential association of SF2 and NL4-3 Nef proteins with the cellular kinase.

Nef's enhancement of infectivity is also viral isolate dependent. To address the possible physiological relevance of our observations, the enhancement of infectivity observed in the presence of Nef was determined for both SF2 and NL4-3. For this purpose, the infectivities of HIV-1 SF2 and HIV-1 NL4-3 were compared with those of similar viruses in which a frameshift mutation was introduced in the *nef* open reading frame. While SF2 and NL4-3 had similar infectivities, for HIV-1 SF2 there was a 38-fold enhancement of infectivity in the presence of Nef (Fig. 4). By contrast, for NL4-3 only a threefold differ-

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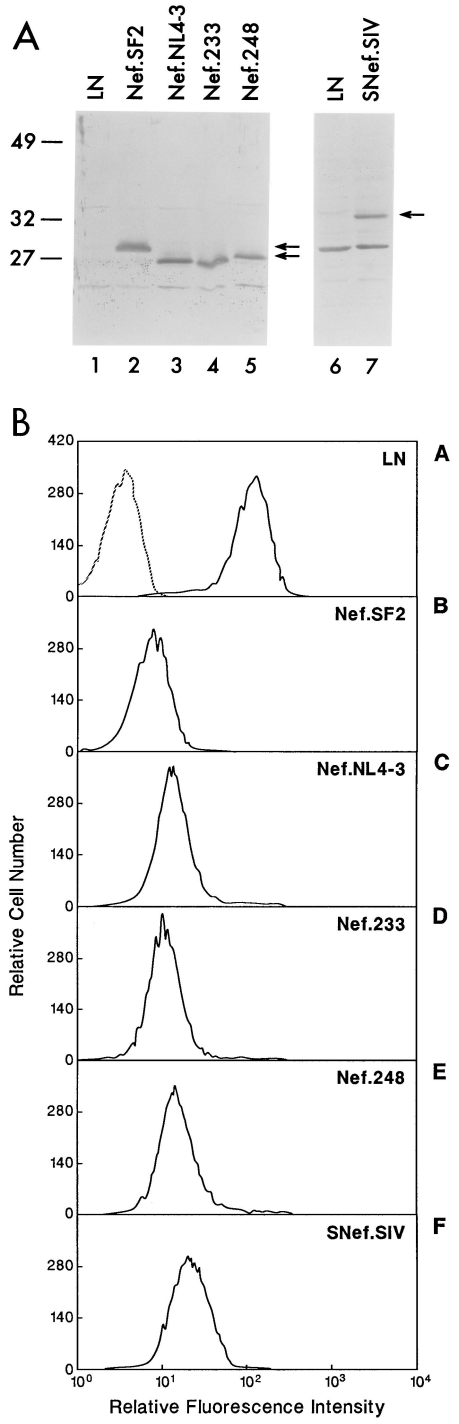

FIG. 1. Expression and functional analysis of Nef isolates in HuT 78 human T cells. Nef was expressed in HuT 78 cells via retrovirus-mediated gene transfer essentially as previously described (4, 5). (A) Western blot analysis of Nef expression. Equal amounts of total cellular proteins (100 μg) from HuT 78 cells expressing different Nef isolates were separated by SDS-PAGE and analyzed by Western blotting with a rabbit anti-HIV-1 Nef (lanes 1 to 5) or a rabbit anti-SIV Nef (lanes 6 and 7) antiserum as previously described (4, 5). Lane 1, HuT 78/LN (negative control); lane 2, HuT 78/LNefSN.SF2; lane 3, HuT 78/LNefSN.Nl4-3; lane 4, HuT 78/LNefSN.233; Lane 5, HuT 78/LNefSN.248; lane 6, HuT 78/LN; lane 7, HuT 78/LSNefSN.SIV. Molecular mass markers (in kilodaltons) are indicated on the left. (B) Flow cytometric analysis of CD4 surface expression. Graph A, HuT 78/LN cells stained with isotype-matched negative control monoclonal antibody (dotted line) or phycoerythrin-conjugated anti-CD4 monoclonal antibody (solid line) (both monoclonal antibodies were purchased from Exalpha, Boston, Mass.); graphs B to F, cells stained with phycoerythrin-conjugated anti-CD4 monoclonal antibody (HuT 78/LNefSN.SF2 [graph B], HuT 78/LNefSN.NL4-3 [graph C], HuT 78/LNefSN.233 [graph D], HuT 78/LNefSN.248 [graph E], and HuT 78/LSNefSN.SIV [graph F]). Flow cytometric analysis was done essentially as described before (4, 22).

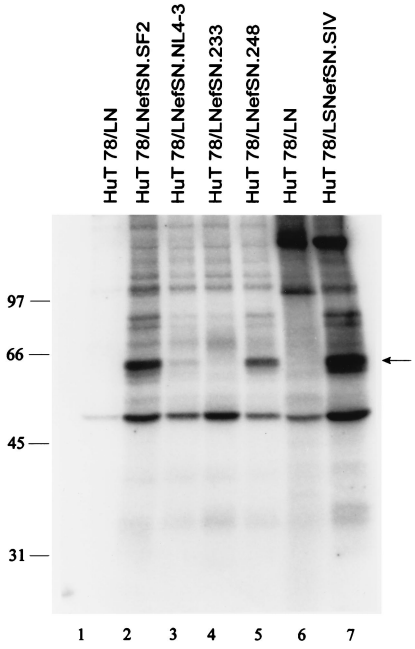

FIG. 2. Nef association with a cellular serine/threonine kinase is isolate dependent. Cell lysates from the HuT 78 cells (4×10^6) expressing Nef described in the legend to Fig. 1 were immunoprecipitated in parallel with rabbit anti-HIV-1 Nef (lanes 1 to 5) or rabbit anti-SIV Nef (lanes 6 and 7) antiserum, and the immunoprecipitates were used for in vitro kinase assay. HuT 78/LN was used as a negative control. Molecular mass markers (in kilodaltons) are indicated on the left. The arrow indicates a 62-kDa protein phosphorylated by the serine/threonine kinase associated with Nef as described by Sawai et al. (29, 30). The in vitro kinase assay was performed essentially as previously described (29, 30). The relative intensities of the 62-kDa phosphoprotein determined with a PhosphorImager (Molecular Dynamics model 425F) for lanes 1 to 7 were 1, 32, 2, 1.2, 15, 1.5, and 69, respectively.

ence in infectivity in the presence of Nef was observed (Fig. 4). These results suggest that Nef's enhancement of infectivity is viral isolate dependent and that the association of Nef with the serine/threonine kinase might be important for the infectivity enhancement observed in some viruses containing Nef.

Nef has been shown to be important for virus replication and pathogenesis (2, 12, 14, 16, 25). However, it is unclear how Nef functions or how Nef functions are regulated. Nef has been found associated with a cellular serine/threonine kinase (29, 30). This represented the first clear evidence that Nef physically interacts with cellular proteins and suggested how Nef might influence viral and cellular functions. However, the correlation between Nef-associated kinase activity and Nef functions has not been established. Our results suggest a possible correlation between the association of Nef with the serine/threonine kinase and an enhancement of infectivity.

Although the identity of the Nef-associated kinase remains to be established, it has been suggested that the association of Nef with the kinase may be important for in vivo pathogenesis (31). Among the five functional isolates of Nef tested, only HIV-1 SF2 and 248 and SIVmac239 Nef proteins were found to efficiently associate with the kinase. These results show that under our experimental conditions the association of Nef with

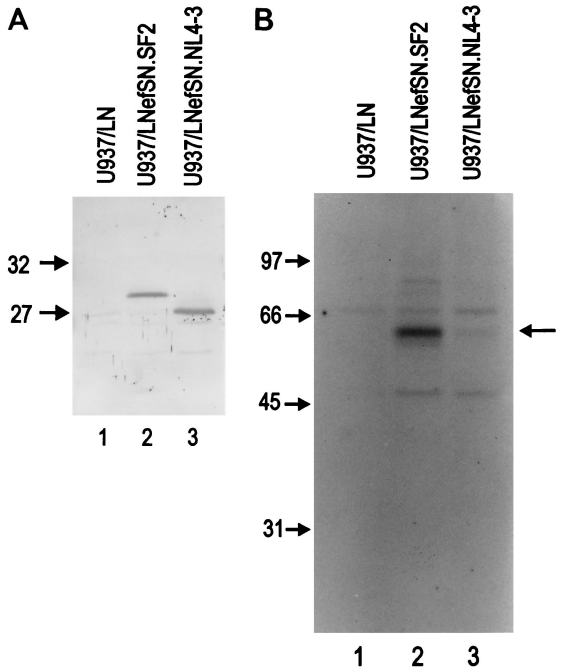

FIG. 3. Failure of Nef to associate with the serine/threonine kinase is cell type independent. (A) Western blot analysis of Nef expression in human U937 cells. Nef was expressed in U937 cells via retrovirus-mediated gene transfer as previously described (4). Equal amounts (100 μg) of total cellular proteins were used for analysis by Western blotting as for Fig. 1. U937/LN cells were used as a control. Molecular mass markers (in kilodaltons) are indicated on the left. (B) In vitro kinase assay. U937/LN cells were used as a control. Molecular mass markers (in kilodaltons) are indicated on the left. The arrow indicates the 62-kDa protein phosphorylated by the Nef-associated cellular kinase as described in the legend to Fig. 2. The relative intensities of the 62-kDa phosphoprotein in lanes 1 to 3 were 1, 40, and 2, respectively.

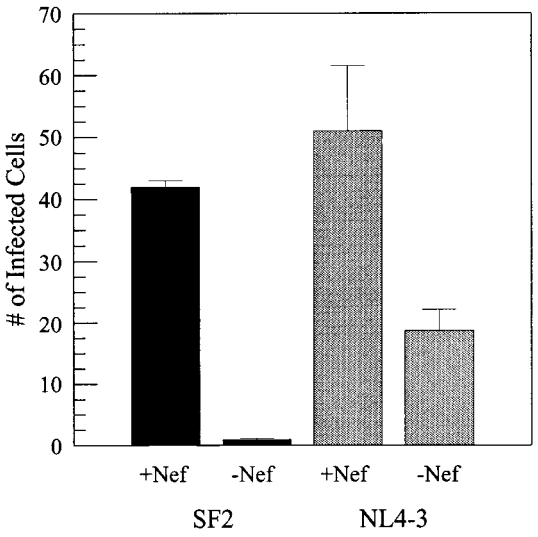

FIG. 4. Nef's enhancement of infectivity is isolate dependent. HIV-1 SF2 or HIV-1 NL4-3 and their respective *nef*-minus virus mutants were produced by transfecting provirus into 293 cells with Lipofectamine. Virus stocks were harvested at 48 h posttransfection and filtered through a 0.45-μm-pore-size filter. Equal amounts of p24 (as determined by using a p24 assay kit [Coulter, Miami, Fla.]) were used to infect HeLa-MAGI cells as previously described (18). Cells were stained at 48 h postinfection with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and blue cells were counted as an indicator of infected cells. Each bar represents the average of three determinations.

the cellular kinase is isolate dependent. However, it is also possible that the NL4-3 and 233 Nef isolates bind to but are unable to activate this cellular kinase in the in vitro kinase assay.

There has been a significant amount of controversy surrounding Nef function. The experimental evidence obtained with animal models suggests an important role for Nef in pathogenesis, disease progression, and the maintenance of high virus loads (2, 14, 16). In contrast, there is no consensus concerning which in vitro functions of Nef translate into the phenotypes observed in vivo. On the basis of the results presented in this article, some of the discrepancies in the literature can now be attributed to intrinsic functional and structural differences between Nef isolates. In the future, broad generalizations with respect to Nef function will benefit from a careful analysis of several different isolates.

We thank M. Emerman for the HeLa-MAGI cells, L. Ratner and R. Desrosiers for the rabbit anti-HIV Nef and anti-SIV Nef antisera, Richard Ashmun for assistance with the flow cytometric analysis, and J. Wineland for technical support. We also thank M. Roussel, Suk-Hee Lee, J. Downing, M. Chien, J. Douglas, J. Foster, and E. O'Neill for their comments on the manuscript. The HuT 78 and U937 cell lines were obtained from the AIDS Research and Reference Reagent Program.

This work was supported by grant AI-33331 (J.V.G.), by Cancer Center Support (CORE) grant CA-21765 from the National Cancer Institute, and by the American Lebanese Syrian Associated Charities (ALSAC) of St. Jude Children's Research Hospital. T.L. is supported by postdoctoral training grant AI-07372 from the National Institute of Allergy and Infectious Diseases.

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