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Selective Restriction of Nef-Defective Human Immunodeficiency Virus Type 1 by a Proteasome-Dependent Mechanism[▽]

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The Nef protein enhances human immunodeficiency virus type 1 (HIV-1) infectivity by facilitating an early postentry step in the virus life cycle. We report here that the addition of MG132 or lactacystin, each a specific inhibitor of cellular proteasome activity, preferentially enhances cellular permissiveness to infection by Nef-defective versus wild-type HIV-1. Pseudotyping by the glycoprotein of vesicular stomatitis virus rendered Nef-defective HIV-1 particles minimally responsive to the enhancing effects of proteasome inhibitors. These results suggest that Nef enhances the infectivity of HIV-1 particles by reducing their susceptibility to proteasomal degradation in target cells.

Specifically encoded by primate lentiviruses, the accessory protein Nef promotes human immunodeficiency virus type 1 (HIV-1) infection by a poorly defined mechanism. Nef-defective particles have been found to be significantly less infectious than wild-type HIV-1 particles when tested in single-cycle infection assays (2, 9, 15, 21). The expression of Nef in the virus-producing cell is required for infectivity enhancement, indicating that Nef modifies the virion during particle assembly or maturation (2). Nef-defective HIV-1 particles fuse efficiently with cells (5, 10, 23), though one recent study reported a defect in the incorporation of envelope glycoprotein in Nef-defective HIV-1 particles (20). Nef-defective virions are impaired for reverse transcription in target cells despite containing normal levels of viral genomic RNA and a normal quantity of active reverse transcriptase enzyme (2, 8, 21). Nef-defective HIV-1 particles resemble wild-type particles both structurally and biochemically; however, some biochemical effects of Nef have been observed in HIV-1 particles. Nef promotes the incorporation of a p21-activated protein kinase family member (17, 19), and Nef-defective HIV-1 particles appear to have reduced levels of cholesterol (28). Despite the lack of an apparent effect of Nef on HIV-1 entry, the requirement for Nef in HIV-1 infection can be markedly reduced by the pseudotyping of HIV-1 cores by the glycoproteins of heterologous enveloped viruses that depend on endosomal acidification for fusion (1, 7, 14). This finding suggests that rerouting the viral core to an endocytic entry pathway bypasses an intracellular restriction selective for Nef-defective HIV-1. Nef is incorporated into HIV-1 particles (3, 18, 25, 26), and a fraction of the virion-associated Nef protein copurifies with HIV-1 cores (12); however, a role of particle-associated Nef protein has not been established. In the present study, we sought to test the hypothesis that Nef-defective HIV-1 particles are specifically restricted by a proteasome-dependent mechanism in target cells.

Proteasome inhibitors selectively enhance infection by Nef-defective HIV-1. Previous studies have established that HIV-1 infection can be enhanced by treatment of cells with chemical inhibitors of the proteasome (22, 24). Such inhibitors likely protect the viral core from degradation in target cells. To determine the relative susceptibility of wild-type and Nef-defective HIV-1 particles to intracellular restriction by the proteasome, we pretreated cultures of HeLa-P4 cells (6), an HIV-1 reporter cell line, with MG132 (25 μM) for 1 h and titrated wild-type and Nef-defective HIV-1 particles on the cells. For these experiments, two similar HIV-1 clones, designated R7 and R8, were tested with their Nef-defective variants encoding a nonsense (i.e., frameshift) mutation in the Nef open reading frame. Viruses were produced by the transfection of 293T cells as previously described (1). Virus and drug were removed 2 h after inoculation, and the cultures were replenished with fresh medium. Two days later, HIV-1-infected cells were detected by staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and quantified. Infectivity was determined as the number of infected cells per nanogram of p24 in the viral inocula. Nef enhanced HIV-1 infectivity at least 20-fold (Fig. 1A and B) in control cultures lacking proteasome inhibitors. The addition of MG132 further enhanced wild-type HIV-1 infectivity, but less than twofold (Fig. 1A). Previous studies reported a somewhat greater enhancement of wild-type HIV-1 infection by MG132, which is likely dependent on the precise infection conditions (22, 24). By contrast to the case for the wild-type virus, infection by Nef-defective HIV-1 was enhanced more than 20-fold by the addition of MG132 (Fig. 1A). The proteasome inhibitor thus reduced the dependence of HIV-1 infection on Nef from 20-fold to less than 2-fold (Fig. 1B).

MG132 is a reversible inhibitor of the 26S proteasome that also exhibits some inhibitory activity toward endosomal proteases. By contrast, lactacystin is highly specific for proteasomes that inhibit by an irreversible mechanism. Like that with MG132, the pretreatment of cells with lactacystin preferentially enhanced infection by Nef-defective HIV-1 (Fig. 1C and D). As a control, we also tested the cathepsin inhibitor E-64, which exhibited only a slight inhibitory effect that appeared to

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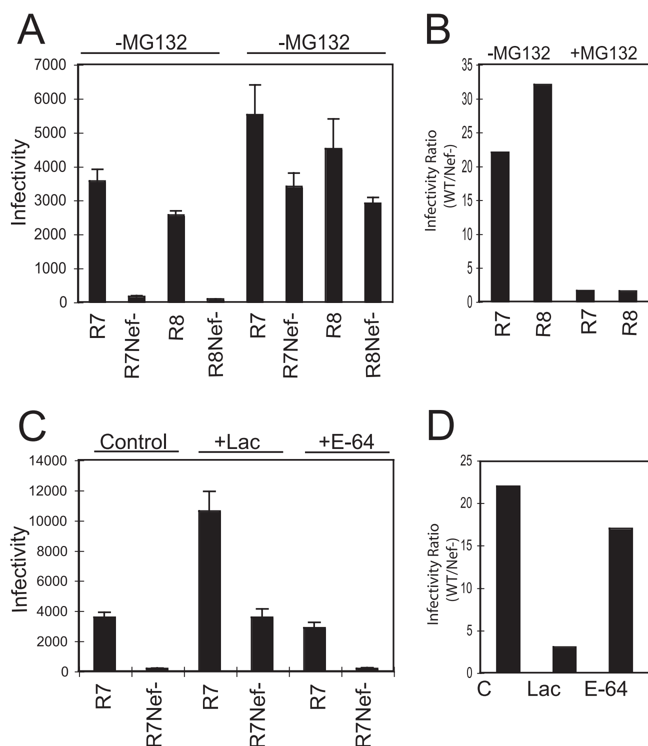

FIG. 1. Inhibition of proteasome activity preferentially enhances cellular susceptibility to infection by Nef-defective HIV-1. (A and C) HeLa-P4 cell cultures, preincubated for 1 h with the indicated inhibitor, MG132 (25 μM), lactacystin (25 μM), or E-64 (40 μM), were inoculated with dilutions of wild-type (R7 and R8) HIV-1 or the Nef-defective mutants (R7Nef$^-$ and R8Nef$^-$). Viruses and drugs were removed 2 h postinoculation, and the cultures were replenished with fresh medium. Cultures were fixed and stained with X-Gal 2 days later, and the infected cells were quantified. Infectivity is expressed as the number of X-Gal-positive cells per nanogram of p24 in the inoculum. Panels B and D show the ratios of wild-type to Nef-defective HIV-1 infectivity determined in the presence (+) and absence (−) of the inhibitors. Error bars indicate standard deviations.

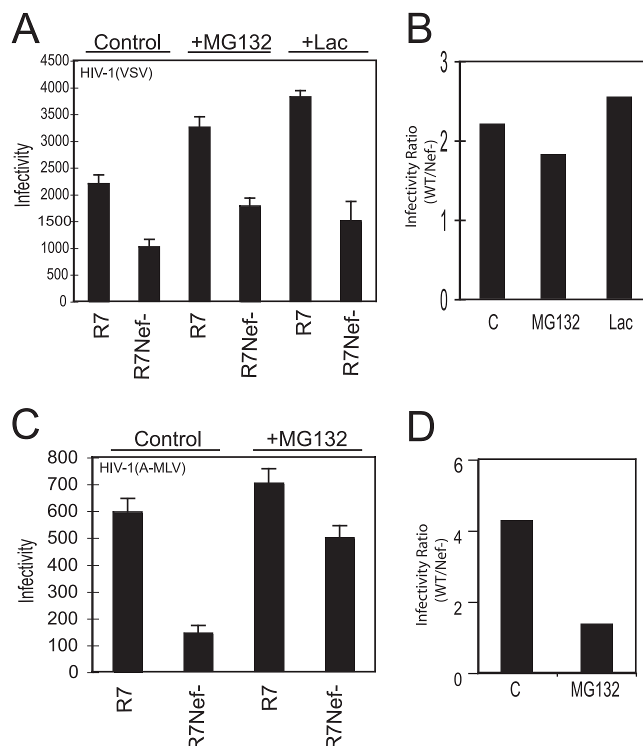

FIG. 2. Effects of MG132 on the infectivity of pseudotyped HIV-1 particles. (A) HIV-1(VSV) pseudotyped particles were produced by the transfection of 293T cells with Env-defective variants of the indicated proviral DNAs and the VSV-G expression vector pHCMV-G (27). (C) HIV-1(A-MLV) particles were produced by cotransfection of Env-defective proviral DNAs with the amphotropic murine leukemia virus Env expression vector pSV-A-MLV-env (13). Panels B and D show the ratios of wild-type/Nef-defective HIV-1 infectivity corresponding to the results in panels A and C, respectively. Error bars indicate standard deviations.

be specific for wild-type HIV-1 (Fig. 1C and D). We conclude that the inhibition of proteasome activity preferentially enhances cellular permissiveness to Nef-defective HIV-1.

MG132 has a similar effect on the infectivity of Nef$^+$ and Nef$^-$ HIV-1 particles pseudotyped by VSV-G. Previous studies have demonstrated that the pseudotyping of HIV-1 particles by the vesicular stomatitis virus glycoprotein (VSV-G) relieves the requirement for Nef in HIV-1 infection (1, 14), probably by targeting HIV-1 entry to an endocytic entry pathway. To further probe the hypothetical link between the preferential infectivity enhancement of Nef-defective HIV-1 by proteasome inhibitors and Nef-dependent HIV-1 infection, we tested the effects of MG132 on infection by Nef$^+$ and Nef$^-$ HIV-1 particles that were pseudotyped by VSV-G [HIV-1(VSV)]. Virus particles were produced by transfection as previously described (1). The results revealed that MG132 enhanced the infectivity of HIV-1(VSV) particles less than twofold under these experimental conditions, irrespective of the expression of Nef (Fig. 2A and B).

By contrast to HIV-1(VSV) pseudotypes, HIV-1 particles bearing the envelope glycoproteins of the amphotropic murine leukemia virus (A-MLV) [HIV-1(A-MLV)] remain dependent on Nef for HIV-1 infection (2, 16). We therefore tested the effects of MG132 on Nef$^+$ and Nef$^-$ HIV-1(A-MLV) pseudotyped particles. Like the case for nonpseudotyped HIV-1, the addition of MG132 prior to inoculation substantially reduced the dependence of infection by the HIV-1(A-MLV) particles on Nef (Fig. 2C and D). These results indicate that the specific enhancement of infection by Nef-defective HIV-1 by MG132 is correlated with the degree to which the infection depends on Nef.

Implications for the mechanism of Nef-dependent enhancement of HIV-1 infectivity. The results in this study reveal a link between the antiviral activity of the proteasome in target cells and the impaired infectivity of Nef-defective HIV-1 particles. We conclude that Nef enhances HIV-1 infectivity by modifying the virion during particle assembly so as to render it less susceptible to proteasomal degradation in target cells. The mechanism by which Nef promotes HIV-1 resistance to the proteasome may be direct or indirect. Infectivity enhancement by Nef requires its expression in the virus-producing cell, indicating that Nef functionally modifies the virion. The presence of Nef within HIV-1 particles and its localization to the viral core suggest that the functional modification may be the presence of Nef itself. In this scenario, the association of Nef with the viral

core may directly protect it from degradation by the ubiquitin-proteasome system in target cells. However, a recent study has reported a mutant Nef protein that enhances infectivity but is not efficiently incorporated into virions, suggesting that the particle association of Nef is not required for optimal HIV-1 infectivity (11). Nef could also modify the virion by inhibiting the ubiquitylation of one or more virion proteins, leading to reduced recognition by proteasomes in target cells. Alternatively, Nef could promote intracellular trafficking of the viral core, allowing it to bypass a hypothetical proteasomal antiviral compartment. Consistent with the latter hypothesis are the observation that pseudotyping by VSV-G relieves the requirement for Nef in HIV-1 infection, as does the treatment of target cells with specific inhibitors of the actin cytoskeleton (4). Although the findings in the present study appear to be at odds with reports that Nef enhances HIV-1 infectivity by a mechanism involving lipid rafts and cholesterol incorporation (28, 29), it is theoretically possible that a raft protein is specifically incorporated into HIV-1 particles, where it acts to protect the incoming viral core from proteasomal destruction in the cytoplasm. Alternatively, Nef may downregulate expression and, thus, HIV-1 incorporation of a cellular factor that targets the core to proteasomes. Additional studies will be required to definitively identify the molecular defect in Nef-defective HIV-1 particles responsible for the enhanced sensitivity to proteasomal interference. Irrespective of the mechanism, the demonstration that Nef is capable of protecting incoming HIV-1 particles from intracellular restriction by the proteasome suggests that other viruses may also have specific mechanisms to evade proteasomal degradation during early stages of infection.

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