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Nef Enhances HIV-1 Infectivity via Association with the Virus Assembly Complex

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Abstract

The HIV-1 accessory protein Nef enhances virus infectivity by facilitating an early post-entry step of infection. Nef acts in the virus producer cell, leading to a beneficial modification to HIV-1 particles. Nef itself is incorporated into HIV-1 particles, where it is cleaved by the viral protease during virion maturation. To probe the role of virion-associated Nef in HIV-1 infection, we generated a fusion protein consisting of the host protein cyclophilin A (CypA) linked to the amino terminus of Nef. The resulting CypA-Nef protein enhanced the infectivity of Nef-defective HIV-1 particles and was specifically incorporated into the virions via association with Gag during particle assembly. Pharmacologic or genetic inhibition of CypA-Nef binding to Gag prevented incorporation of CypA-Nef into virions and inhibited infectivity enhancement. Our results indicate that infectivity enhancement by Nef requires its association with a component of the assembling HIV-1 particle.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses encode accessory proteins in addition to the prototypic *gag, pol* and *env* open reading frames. One of these, Nef, is found only in primate lentiviruses and is required for efficient HIV-1 replication in primary $CD4^+$ T cells, macrophages, and some T cell lines (Miller et al., 1994; Spina et al., 1994). Inactivation of Nef in HIV-1 and the related simian immunodeficiency virus (SIV) leads to attenuated replication and delayed onset of disease in the infected host (Kestler III et al., 1991; Learmont et al., 1999). Expression of Nef also has profound effects on cells, including downmodulation of cell surface CD4 and MHC class I expression as well as enhancement of T cell activation (Baur et al., 1994; Garcia and Miller, 1991; Kestler III et al., 1991; Schwartz et al., 1996).

Nef-defective viral particles are 4–40 fold less infectious than wild type HIV-1 in single cycle infection assays (Aiken and Trono, 1995; Chowers et al., 1994; Miller et al., 1994). Despite intensive studies, the molecular mechanism by which Nef enhances HIV-1 infectivity remains unclear. Nef does not markedly enhance the efficiency of HIV-1 entry into cells, nor does it appear to modulate the intrinsic stability of the HIV-1 capsid shell that surrounds the viral ribonucleoprotein complex (Cavrois et al., 2004; Day, Munk, and Guatelli, 2004; Forshey and Aiken, 2003; Tobiume et al., 2003). Nef-defective HIV-1 particles contain normal quantities

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of reverse transcriptase and enter cells efficiently but are impaired for reverse transcription in target cells (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995). Wild type and Nef-defective virions exhibit similar protein compositions (Miller et al., 1995; Schwartz et al., 1995), except when produced in cells expressing CD4, where Nef-induced CD4 down regulation promotes incorporation of the viral envelope glycoproteins into HIV-1 particles (Lama, Mangasarian, and Trono, 1999; Lundquist, Zhou, and Aiken, 2004; Ross, Oran, and Cullen, 1999). Expression of Nef *in trans* in the virus-producing cell, but not in the target cell, complements the impaired infectivity of Nef-defective HIV-1 particles (Aiken and Trono, 1995; Miller et al., 1995). A recent study shows that the host protein dynamin 2 is required for HIV-1 infectivity enhancement by Nef (Pizzato et al., 2007), and Nef has been reported to promote incorporation of cholesterol into HIV-1 particles (Zheng et al., 2003). A notable difference between wild type and Nef-defective virions is the presence of Nef itself in HIV-1 particles (Bukovsky et al., 1997; Pandori et al., 1996; Welker et al., 1996), where it is cleaved by the viral protease. Collectively, these observations suggest that Nef may promote HIV-1 infection by modulating the budding virion to facilitate uncoating, intracellular transport, or reverse transcription in target cells.

Incorporation of Nef into HIV-1 particles has been correlated with infectivity enhancement. Incorporation of Nef into HIV-1 particles depends on its association with cell membranes: mutations that prevent its myristoylation, as well as those in its amino terminal basic domain, result in impaired virion incorporation as well as reduced HIV-1 infectivity (Aiken and Trono, 1995; Welker et al., 1998). Mutations in a C-terminal region of Nef were also found to inhibit virion incorporation of Nef and HIV-1 infectivity (Zheng et al., 2003). However, a limitation of genetic loss-of-function studies is the possibility of pleiotropic effects of the mutations; such studies must therefore be interpreted with caution. Thus, a genetic correlation of infectivity enhancement with the presence of Nef in HIV-1 particles does not necessarily establish a role of virion-associated Nef in HIV-1 infection.

The host cell protein cyclophilin A (CypA) also promotes HIV-1 infection. CypA is a peptidylprolyl isomerase and the target of the immunosuppressive drug cyclosporine A (CsA). CsA inhibits HIV-1 replication in primary T cells and established T cell lines (Bartz et al., 1995; Karpas et al., 1992; Wainberg et al., 1988). CypA is incorporated into HIV-1 particles via association with the CA region of Gag (Franke, Yuan, and Luban, 1994; Thali et al., 1994a). Mutations in Gag that inhibit CypA binding also reduce HIV-1 infectivity, as does production of HIV-1 particles in the presence of CsA (Braaten, Franke, and Luban, 1996; Franke, Yuan, and Luban, 1994; Thali et al., 1994a). These findings suggested that incorporation of CypA into HIV-1 particles is necessary of optimal infectivity. However, recent studies using CypA-deficient cells have shown that the decrease in HIV-1 infectivity observed when virions are produced in the presence of CsA is independent of the CypA-Gag interaction (Hatziioannou et al., 2005; Sokolskaja, Sayah, and Luban, 2004). Thus, it appears that the engagement of the viral capsid by target cell CypA following HIV-1 entry promotes HIV-1 infection.

In the present study, we exploited the ability of CypA to be specifically incorporated into HIV-1 particles to examine the role of producer cell Nef in HIV-1 infectivity. By analyzing the effects of an engineered CypA-Nef fusion protein, we obtained novel evidence that association of Nef with the assembling virion is necessary for enhancement of HIV-1 infectivity by the viral protein.

Results

Expression of a CypA-Nef fusion protein

To regulate incorporation of Nef into HIV-1 particles, we constructed a cDNA encoding a fusion protein consisting of CypA fused to the amino terminus of HIV-1 Nef (Fig. 1A). Because Nef is myristylated at its amino terminus and this modification is required for incorporation into HIV-1 particles during their assembly, we expected that virion incorporation of the resulting CypA-Nef fusion protein (CypA-Nef) would require interaction of the CypA region of the molecule with Gag during assembly. Both Nef and CypA-Nef were efficiently expressed in transfected 293T cells, as detected by immunoblotting using a Nef-specific antiserum (Fig. 1B). As a control for CypA-Nef, we also generated CypA expression plasmid. To circumvent the interfering immunoblotting signal from the endogenous CypA, HA-tagged CypA and HA-tagged CypA-Nef were also constructed. These proteins were expressed under control of the CMV promoter and were produced at similar levels (Fig. 1B). Immunoblot analysis of endogenous CypA and β-actin levels demonstrated that overexpression of Nef, CypA-Nef, HA-CypA, or HA-CypA-Nef did not significantly alter the endogenous level of CypA in 293T cells (data not shown).

CypA-Nef enhances the infectivity of Nef-defective HIV-1

To determine whether CypA-Nef can functionally complement the reduced infectivity of Nef-defective particles, we cotransfected 293T cells with a Nef-defective HIV-1 provirus and plasmids encoding CypA-Nef or Nef and analyzed the infectivity of the resulting virus stocks by titration on P4 reporter cells. The P4 cell line is a Hela cell derivative that has been modified to express human CD4 and contains a stably-transfected Tat-responsive lacZ reporter. Infection of these cells with HIV results in expression of Tat and expression of β-galactosidase, thus allowing quantitation of infected cells following visualization by staining with X-gal. The infectivity of Nef-defective HIV-1 particles was enhanced by CypA-Nef by approximately three-fold, to a level approximately one-half that of wild type HIV-1 but similar to the infectivity of Nef-defective HIV-1 particles complemented by a Nef-expression plasmid (Fig. 2A). To determine whether the Nef portion of CypA-Nef is necessary for infectivity enhancement, we produced Nef-defective HIV-1 particles by cotransfection of 293T cells with a vector encoding only CypA. The results indicated that exogenous CypA alone did not enhance the infectivity of Nef-defective virions (Fig. 2B). Viruses were also produced by cotransfection of Nef-defective HIV-1 provirus with plasmids encoding the HA-tagged proteins. The resulting viruses were then assayed for infectivity. HA-CypA-Nef enhanced the infectivity of Nef-defective HIV-1 while HA-CypA did not (Fig. 2B). Together, these data suggest that CypA-Nef enhances infectivity by a mechanism that depends on the Nef portion of the fusion protein. Therefore, the strategy could be useful for testing the requirement for association of Nef with the assembling virion in HIV-1 infectivity enhancement.

To examine whether CypA-Nef functionally substitutes for Nef in HIV-1 infection, we asked whether this fusion protein could also enhance the infectivity of wild type HIV-1 particles. To test this, cells were cotransfected with wild type and Nef-defective HIV-1 proviruses together with Nef or CypA-Nef expression vectors, and the resulting virus stocks were assayed for infectivity. CypA-Nef and Nef enhanced the infectivity of wild type HIV-1 only slightly, while both Nef and CypA-Nef enhanced the infectivity of Nef-defective HIV-1 by three-fold (Fig. 2C). These results indicate that CypA-Nef expression during HIV-1 particle production specifically complements the infectivity impairment resulting from the lack of Nef.

CypA-Nef is incorporated into the virion and associates with the viral core

To test whether CypA-Nef is incorporated into HIV-1 particles, we purified the virions from the culture supernatants and prepared lysates of the transfected 293T cells. Analysis of protein

immunoblots revealed the presence of Nef and CypA-Nef in viral particles (Fig. 2D). Two major bands were observed in the particles containing CypA-Nef, corresponding in size to the full-length fusion protein and a product equivalent in mobility with the large fragment of Nef resulting from cleavage of Nef by the viral protease. Thus both cleaved and uncleaved forms of CypA-Nef were detected in HIV-1 particles, consistent with the known ability of virion-associated Nef to be cleaved by the viral protease (Bukovsky et al., 1997;Pandori et al., 1996;Welker et al., 1996). We have not yet determined whether cleavage of CypA-Nef is mediated by the viral protease or is cleaved at the same position as Nef itself. In some experiments, the level of virion-associated CypA-Nef was significantly greater than that of Nef when provided *in trans* by cotransfection, suggesting that the fusion protein may be more efficiently incorporated into HIV-1 particles than Nef itself. We conclude that CypA-Nef is efficiently incorporated into HIV-1 particles.

In previous studies, our laboratory reported that Nef, though associated with the HIV-1 core, does not modulate the intrinsic stability of the viral core (Forshey and Aiken, 2003; Kotov et al., 1999). To determine whether CypA-Nef had an effect on the yield of cores, which is a measure of their intrinsic stability (Forshey et al., 2002), we purified viral cores from Nef-defective HIV-1 particles produced by cotransfection with Nef or CypA-Nef expression constructs. The CA protein was detected at similar levels in association with cores recovered from the two viruses (Fig. 3A), suggesting that CypA-Nef does not alter the intrinsic stability of the HIV-1 core. Samples of purified cores were then analyzed by immunoblotting for the presence of Nef and CypA-Nef. Both proteins were detected in the purified cores at comparable levels, suggesting that CypA-Nef, like Nef, associates with the mature HIV-1 core (Fig. 3B). This intravirion association is likely dependent on the Nef region of CypA-Nef, since both full-length CypA-Nef and the cleaved fragment of Nef were copurified with the core structures, and the bulk of virion-associated CypA is removed upon purification of HIV-1 cores ((Welker et al., 2000); our unpublished observations).

Genetic correlation of Nef and CypA-Nef function in HIV-1 infectivity enhancement

Point mutations in Nef have been shown to impair HIV-1 infectivity enhancement (Craig, Pandori, and Guatelli, 1998; Fackler et al., 2006; Goldsmith et al., 1995; Lundquist et al., 2002). To further probe the issue of whether CypA-Nef enhances the infectivity of Nef-defective HIV-1 particle by a Nef-related mechanism, we constructed four CypA-Nef mutants encoding specific substitutions in the Nef portion of the protein. Nef.4E-4Q and Nef.EKH40AAA were competent for enhancement of HIV-1 infectivity, while HIV-1 encoding Nef.69A/72A and Nef.KEK96AAA were poorly infectious (Fig. 4A). When we cotransfected CypA-Nef plasmids that contain the same mutations together with Nef-defective provirus, we observed that the mutant proteins enhanced the infectivity of Nef-defective virions differently. CypA-Nef.4E-4Q and CypA-Nef.EKH40 enhanced the infectivity of Nef-defective virions by 2.5-fold, while CypA-Nef.69A/72A and CypA-Nef.KEK96AAA failed to enhance the infectivity of Nef-defective virions (Fig. 4B). We observed a statistically significant correlation of the infectivity enhancement by CypA-Nef mutants *in trans* and Nef mutants *in cis* (R^2=0.9991; $p<0.05$ by Spearman's rank correlation test; Fig. 4C). Immunoblot analysis confirmed that the mutant CypA-Nef proteins were efficiently incorporated into HIV-1 particles (Fig. 4D). These results suggest that Nef and CypA-Nef employ a common mechanism to enhance the infectivity of Nef-defective virions.

CsA treatment inhibits virion incorporation of CypA-Nef and infectivity enhancement

To determine whether enhancement of HIV-1 infectivity by CypA-Nef requires its association with Gag in the virus-producing cell, we produced viruses by culturing the transfected 293T cells in the presence and absence of CsA to inhibit the binding of CypA to Gag. This approach has been used previously to prevent the incorporation of endogenous CypA into HIV-1

particles. Viruses were harvested and assayed for infectivity and assayed by immunoblotting for levels of CypA-Nef. CsA treatment reduced the infectivity of control Nef-defective virions by approximately three-fold (Fig. 5A), consistent with the well documented ability of CsA to inhibit HIV-1 infectivity when present at the time of virus production (Aiken, 1997;Braaten, Franke, and Luban, 1996;Steinkasserer et al., 1995;Thali et al., 1994b). By contrast, CsA reduced the infectivity of the virions produced by cotransfection of CypA-Nef by about 9-fold, effectively abolishing the positive effect of CypA-Nef on HIV-1 infectivity (Fig. 5A). As a control, we also tested the effects of CsA on Nef-defective virions produced by coexpression of Nef. CsA inhibited the infectivity of this virus by 5-fold (Fig. 5A), consistent with the reported greater sensitivity of wild type vs. Nef-defective HIV-1 to CsA (Aiken, 1998). Immunoblot analysis of the pelleted virions revealed that CsA reduced the level of virion-associated CypA-Nef but not Nef itself (Fig. 5B). Analysis of cell lysates indicated that CsA treatment did not significantly alter the expression of CypA-Nef or Nef (Fig. 5B). Thus, treatment of virus-producer cells inhibited HIV-1 infectivity enhancement by CypA-Nef and its incorporation of the protein into virions. Collectively, these results suggest that enhancement of HIV-1 infectivity by CypA-Nef is dependent on its interaction with the assembling virion and possibly its incorporation into virions.

Mutations in the CypA binding site of Gag binding site diminish both HIV-1 incorporation of CypA-Nef and infectivity enhancement

Incorporation of cellular CypA into HIV-1 particles is also dependent on the sequence of specific loop on the surface of the CA protein (Franke, Yuan, and Luban, 1994; Gamble et al., 1996; Thali et al., 1994a). To further test whether HIV-1 incorporation of CypA-Nef is correlated with infectivity enhancement, we used two well-characterized HIV-1 Gag mutants, G221A and P222A, that inhibit CypA binding. Importantly, the ability of Nef to enhance HIV-1 infectivity is unaffected by either of these Gag mutations (Aiken, 1998). Additional control studies confirmed that the mutations do not impair the ability of *nef*-defective HIV-1 infectivity to be is enhanced by expression of Nef *in trans* (Supplementary Fig. 1). The mutant viruses were produced by cotransfection with the CypA-Nef expression construct, and the resulting virus particles were assayed for infectivity and analyzed by immunoblotting to determine the levels of CypA-Nef incorporation. By contrast to the control virus lacking these mutations, CypA-Nef enhanced the infectivity of the G221A and P222A mutant viruses only slightly (Fig. 6A). The levels of virion-associated CypA-Nef were also decreased in the Gag mutants despite equivalent cellular expression (Fig. 6B).

To further test the requirement for CypA-Nef binding to Gag in HIV-1 infectivity enhancement, we analyzed the effects of a substitution (R55A) in the CypA region of CypA-Nef that interferes with HIV-1 incorporation of CypA (Dorfman et al., 1997). This protein, CypA.R55A-Nef, was expressed in cells at levels similar to wild type CypA-Nef, but its incorporation into HIV-1 particles was markedly reduced and it failed to enhance the infectivity of Nef-defective HIV-1 (Supplemental Fig. 2). Thus, a mutation in the CypA region of CypA-Nef prevented incorporation of the fusion protein into HIV-1 particles and suppressed its ability to enhance HIV-1 infectivity. We conclude that association of CypA-Nef with the assembling viral particle is necessary for infectivity enhancement.

Discussion

Despite significant efforts on the part of several laboratories, the mechanism by which Nef enhances HIV-1 infectivity remains poorly defined. Nef modifies HIV-1 particles in such a way as to facilitate an early post-penetration step in infection. One consistently observed Nef-specific virion modification is incorporation of Nef itself into HIV-1 particles. In this study, we fused CypA to Nef to allowed controlled incorporation of Nef into HIV-1 particles via

association with Gag during particle assembly. CypA-Nef specifically enhanced the infectivity of Nef-defective HIV-1 particles. CypA-Nef was incorporated into the newly formed viral particles; genetic or pharmacologic inhibition of the interaction Gag-CypA-Nef interaction resulted in reduced the ability of CypA-Nef to be incorporated into HIV-1 particles and to enhance virus infectivity. Furthermore, mutations in the Nef that inhibit HIV-1 infectivity also inactivated CypA-Nef, thus demonstrating that the infectivity enhancement by CypA-Nef mutants is correlated with the infectivity enhancement by the same Nef mutants. CypA-Nef was detected in purfied HIV-1 cores, suggesting that the Nef component determines the subviral localization. Collectively, these results indicate that CypA-Nef and Nef enhance the infectivity of Nef-defective virions by a common mechanism.

Our data suggest that interaction of Nef with the assembling HIV-1 particle is required for infectivity enhancement. Mutations in the CypA segment of CypA-Nef, or in Gag, that prevented the interaction of the two proteins also inhibited infectivity enhancement, as did addition of CsA during virus assembly. It is possible that Nef must be incorporated into HIV-1 particles to enhance infectivity, but our data do not definitively establish this as Nef may act prior to virus budding and maturation and incorporation into virions may be a by-product of Nef's accumulation at the site of virus assembly. In a previous study, Fackler and coworkers reported that the 4E4Q Nef mutant enhances HIV-1 infectivity yet is poorly incorporated into HIV-1 particles (Fackler et al., 2006). In attempts to confirm their observations, we have found that the 4E4Q mutant Nef protein is readily detectable in pelleted HIV-1 particles rigorously purified by velocity gradient sedimentation (our unpublished data). In additional studies, we attributed the discrepancy with the Fackler data to the antibody used for detection. Therefore, virion incorporation of Nef is correlated with infectivity enhancement, and a role for virion-associated Nef in HIV-1 infection remains a viable hypothesis.

In this study, we exploited the specific binding of CypA to Gag to target Nef the assembling virion as a fusion with CypA. However, the specific mechanism by which Nef itself engages the virus assembly complex remains unclear. Incorporation of Nef into HIV-1 particles depends on its membrane-binding ability, suggesting that Nef may be incorporated into particles passively by being present at the membrane where the virus buds. A fraction of cellular Nef protein copurifies with detergent-resistant membrane domains known as lipid rafts (Giese et al., 2006; Wang et al., 2000; Zheng et al., 2001). These and other distinct membrane structures have been implicated in HIV-1 assembly (Ding et al., 2003; Holm et al., 2003; Ono and Freed, 2001). It is therefore plausible that Nef localizes to membranes on which HIV-1 assembly occurs. Additional evidence suggests that Nef interacts specifically with internal components of the virion. Nef binds the transframe protein p6* in Gag-Pol, and can interact with both reverse transcriptase and integrase proteins (Ciuffi et al., 2004). Nef also copurifies with isolated HIV-1 cores, suggesting that it relocalizes from the viral membrane to the core during maturation (Kotov et al., 1999). Uncoating of HIV-1 cores in vitro results in dissociation of CA and RT, releasing an uncoated ribonucleoprotein complex with which Nef remains associated (Forshey and Aiken, 2003). Thus, while membrane binding is essential for incorporation of Nef into HIV-1 particles, Nef may perform its function by specifically targeting an internal component of the HIV-1 core.

How would a Nef-mediated virion modification lead to enhanced infectivity? Several biochemical virion modifications have been attributed to Nef, including phosphorylation of MA (Swingler et al., 1997), stimulation of Env incorporation (Schiavoni et al., 2004), and elevation of virion-associated cholesterol and ganglioside GM1 (Zheng et al., 2003; Zheng et al., 2001). Thus, Nef may promote infectivity via multiple effects on virion composition. Inhibition of proteasome activity enhances cellular permissiveness to HIV-1 infection (Schwartz et al., 1998; Wei et al., 2005), and we recently showed that addition of proteasome inhibitors during virus inoculation preferentially enhances the infectivity of Nef-defective

particles, effectively reducing HIV-1 dependence on Nef (Qi and Aiken, 2007). This finding suggests that Nef-defective virions are hypersusceptible to proteasomal interference in the target cell. We hypothesize that a Nef-dependent modification of the assembling virion renders it less susceptible to proteasome-dependent restriction in target cells. Despite the attractiveness of this model, Dueck and coworkers have recently reported evidence suggesting that the stimulatory effect of proteasome inhibitors on HIV-1 infection results from an indirect effect on the cell cycle rather than an antiviral effect of the proteasome (Dueck and Guatelli, 2007). They were also unable to confirm our observation that the Nef-defective HIV-1 infection impairment is relieved by proteasome inhibitors (Qi and Aiken, 2007). We suspect that the latter discrepancy is due to differences in experimental conditions. Though the extent of rescue of the Nef-defective phenotype by proteasome inhibitors varies between individual experiments, we have consistently observed this effect and stand by the conclusions of our previous study. It remains unclear whether the preferential enhancement of Nef-defective HIV-1 by proteasome inhibitors is due to elevated susceptibility of Nef-defective HIV-1 to cytoplasmic degradation or a cell-cycle-dependent selective block to Nef^- particles, and both are possibilities.

Nef may promote proteasomal evasion directly by tethering the viral core it to a cellular trafficking pathway, thereby facilitating intracytoplasmic transport. Nef contains several motifs that are involved in trafficking in the endocytic pathway, and associates with known cellular trafficking molecules and pathways (reviewed in (Bresnahan et al., 1998; Craig, Pandori, and Guatelli, 1998; Erdtmann et al., 2000; Fackler et al., 2006; Greenberg, Iafrate, and Skowronski, 1998; Janvier et al., 2001; Lacaille and Androlewicz, 2000; Mandic et al., 2001). Thus, Nef could promote association of the core with components of the endocytic machinery. Several observations are consistent with this model. First, the impaired infectivity of Nef-defective HIV-1 particles is relieved by pseudotyping by heterologous viral envelopes which target viral entry to an endocytic pathway, such as those of vesicular stomatitis virus and Ebola virus (Aiken, 1997; Chazal et al., 2001; Luo et al., 1998). This not the case for other heterologous Env proteins that do not render HIV-1 infection dependent on endosomal acidification, such as the amphotropic murine leukemia virus (Aiken and Trono, 1995; Miller et al., 1995). Thus, endocytic entry of HIV-1 may physically bypass an intracellular restriction to the Nef-defective viral core. Second, mutations in a dileucine motif in Nef inhibit both CD4 downregulation and infectivity enhancement (Craig, Pandori, and Guatelli, 1998), suggesting that Nef may bind a single cellular factor required for both of these activities. Finally, the infectivity of Nef-defective HIV-1 particle is preferentially enhanced by treatment of target cells with chemicals that depolymerize the cortical actin cytoskeleton (Campbell, Nunez, and Hope, 2004), suggesting that this network forms a barrier to intracytoplamic transport of Nef-defective HIV-1 cores. Despite the attractiveness of the model, the evidence in support of a role of Nef in facilitating intracellular trafficking of the HIV-1 cores is largely circumstantial.

The ability of CypA-Nef to enhance HIV-1 infectivity may facilitate identification of the essential functional regions of Nef. Nef is composed of two discrete domains, a flexible linker domain and a globular core domain (Barnham et al., 1997; Grzesiek et al., 1996; Lee et al., 1996a; Lee et al., 1996b); reviewed in (Geyer, Fackler, and Peterlin, 2001), and thus far it has not been possible to segregate the membrane-binding function of Nef from infectivity enhancement. By targeting Nef to the virion via CypA, it should be possible to identify the minimal Nef determinants of infectivity enhancement from those necessary for its association with the assembling virus.

Materials and Methods

Cells and viruses

293T and HeLa-CD4/LTR-lacZ (P4) cells were cultured in Dulbecco's modified Eagle medium (Cellgro) supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) at 37°C and 5% CO2. The HIV-1 proviral DNA constructs R7 and R7 Nef (Aiken and Trono, 1995) were used for these studies. This viral clone encodes a CXCR4-dependent HIV-1 envelope protein. The Gag mutants G221A and P222A have been described (Aiken, 1998). VSV-G-pseudotyped HIV-1 particles were produced as previously described (Aiken, 1997). Viruses encoding amino acid substitutions in Nef were based on the X4-tropic R9 viral clone and were previously described (Lundquist et al., 2002). Viruses were produced by polyethyleneimine (PEI) transfection of 293T cells (10 μg of plasmid DNA per 5×10^6 cells) (Durocher, Perret, and Kamen, 2002). Where indicated, CsA (Sandoz, Inc.) was added to a final concentration of 5 μg/ml 12h after transfection. One day later, the culture supernatants were harvested and clarified by passing through 0.45-μm pore size syringe filters, and aliquots were frozen at −80°C. The CA contents of the virus stocks were quantified by p24 enzyme-linked immunosorbent assay (ELISA), as previously described. The P4 cell line, a Hela clone engineered to express CD4 and an integrated long terminal repeat (LTR)-*lacZ* reporter cassette, was used to quantify HIV-1 infectivity (Charneau, Alizon, and Clavel, 1992). HIV-1 stocks were serially diluted in culture medium, and samples (0.125 ml) were used to inoculate P4 target cells seeded the day before (20,000 cells per well in 48-well plates). Two hours after inoculation, the cultures were fed with additional medium (0.5 ml) and cultured for another 48 h prior to being stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to detect infected cells. To determine the number of infected cells per well, individual wells were visualized using a charge-coupled device camera equipped with a macro lens, and blue cells were counted using NIH Image software. Infections were performed in triplicate, and only values within the linear range of the infection assay (50–500 blue cells per well) were used to calculate infectivity. Infectivity was calculated as the number of blue cells per ng of p24 added to the well.

Expression plasmids

All expression plasmids in this study were generated in the CMX-PL1 expression vector. Fusion proteins were produced by PCR splice overlap extension. The CypA-Nef cDNA was generated by PCR overlap fusion using the primers 5′-actgtggacaactcgagggtggcaagtggtcaaaaa-3′ (sense) and 5′-tttttgaccacttgccaccctcgagttgtccacagt-3′ (antisense). The HA tags and R55A mutation were also introduced by PCR. The primer for HA-CypA was: 5′-aaggatccaccatgtacgatgttccagattacgctcttgtcaaccccaccgtgttc-3′. The primers for generating the R55A mutation in CypA were: 5′-tcctgctttcacgcaattatcccggggtttatg-3′ (sense) and 5′-cataaaccccgggataattgcgtgaaagcagga-3′ (antisense). PCR fragments were inserted into the CMV promoter-based expression plasmid CMX-PL1 using the BamHI and PstI restriction sites. The constructs were sequenced to confirm the presence of the desired open reading frame and the absence of undesired mutations.

Isolation of HIV-1 cores

Native HIV-1 cores were isolated from concentrated virions as previously described (Forshey et al., 2002; Kotov et al., 1999). Fractions containing HIV-1 cores were identified by quantifying CA by p24 ELISA and by determining the densities of the gradient fractions by refractometric analysis. Cores present in the peak fractions were concentrated by ultracentrifugation at 100,000 ×g for 30 min at 4°C following dilution into STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM EDTA) to reduce the solution density.

Protein analyses

Cell lysates, viral pellets, and pelleted HIV-1 cores were subjected to electrophoresis on 4–20% polyacrylamide gradient gels containing SDS (Bio-Rad Laboratories), and proteins were transferred electrophoretically to nitrocellulose membranes. Protein blots were probed with antibodies to individual HIV-1 proteins, including rabbit polyclonal anti-CA and anti-Nef (from D. Trono), anti-CypA (Upstate Biotechnology, Inc.), and rat monoclonal to HA (Roche Applied Science). Following incubation with infrared dye-conjugated secondary antibodies, protein bands were detected using a LI-COR Odyssey imaging system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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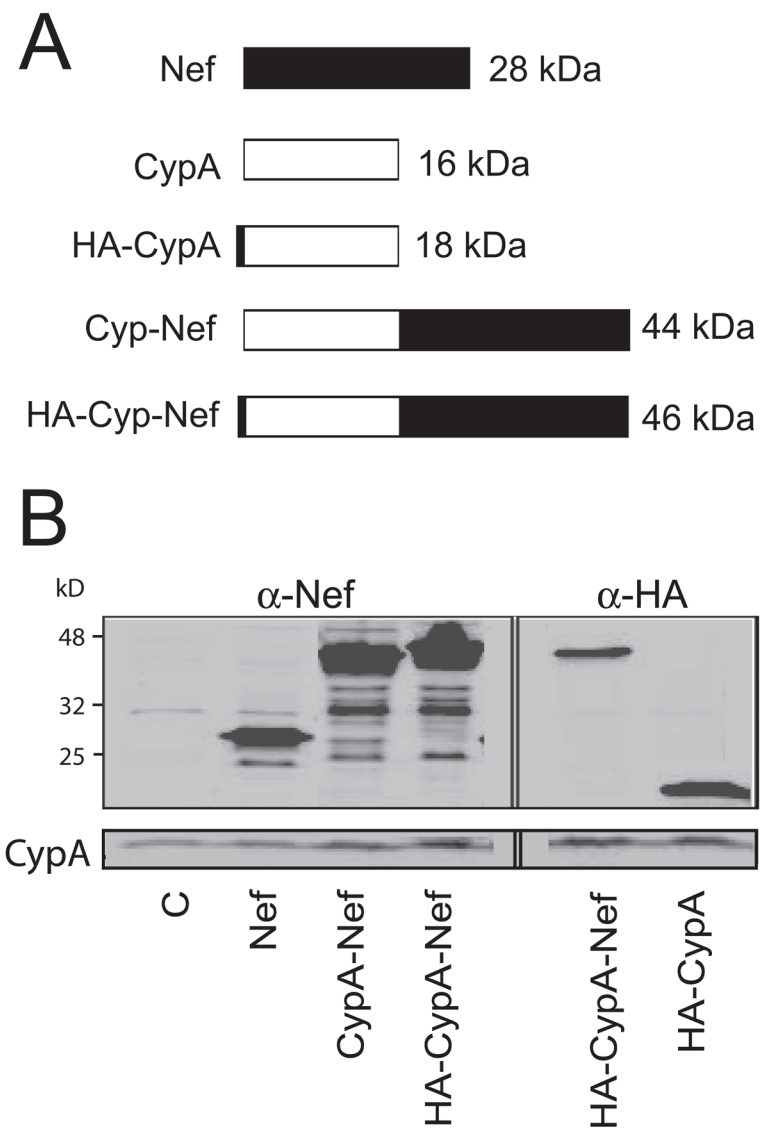

Fig. 1.

Protein constructs used in this study and their expression in 293T cells. (A) Schematic of the five proteins used in this study. (B) Expression of the proteins in 293T cells. Cell lysates were analyzed by immunoblotting. Proteins in the left panel were detected with rabbit antibody for Nef, and proteins in the right panel were detected with rat antibody for HA tag. Abbreviations are as follows: C, control vector; WT, wild type HIV-1; CypA, cyclophilin A; HA: hemagglutinin epitope tag; CA, viral capsid protein.

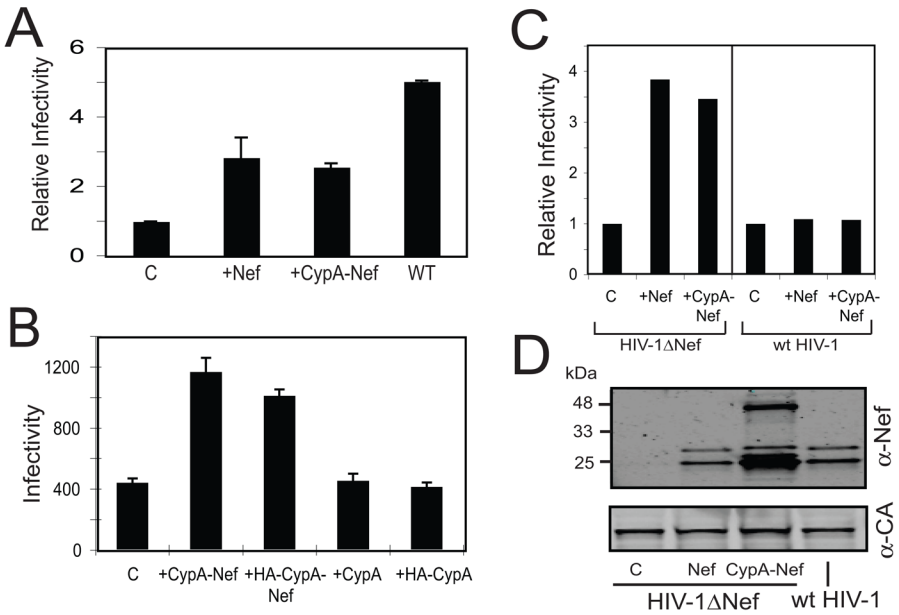

Fig. 2.

CypA-Nef is incorporated into Nef-defective HIV-1 particles and enhances their infectivity. Viruses were produced in 293T cells by cotransfection with a Nef-defective HIV-1 provirus together with plasmids encoding the indicated proteins. Wild type (WT) HIV-1 was tested as a control. Virus supernatants were harvested and assayed for p24 and titrated on P4 indicator target cells. Infectivity was determined as the number of infected cells per ng of p24 in the respective inocula. (A) Shown are the relative infectivity values obtained from 3 independent experiments, with error bars representing one standard deviation. Infectivity values were normalized to that of the control Nef-defective virus. (B) The infectivity results from a representative single experiment. Error bars represent the standard deviation of triplicate assays of each virus. (C) Wild type and Nef-defective virions were produced by cotransfection with constructs encoding Nef or CypA-Nef. The resulting supernatants were assayed for infectivity on P4 indicator cells. Infectivity is shown relative to the corresponding Nef-defective and wild type control viruses to illustrate the differential effects of expression of Nef and CypA-Nef on the two viruses. (D) Immunoblot analysis of pelleted virus particles with antibodies specific for Nef and CA. The results shown in this figure are representative of 2 independent experiments.

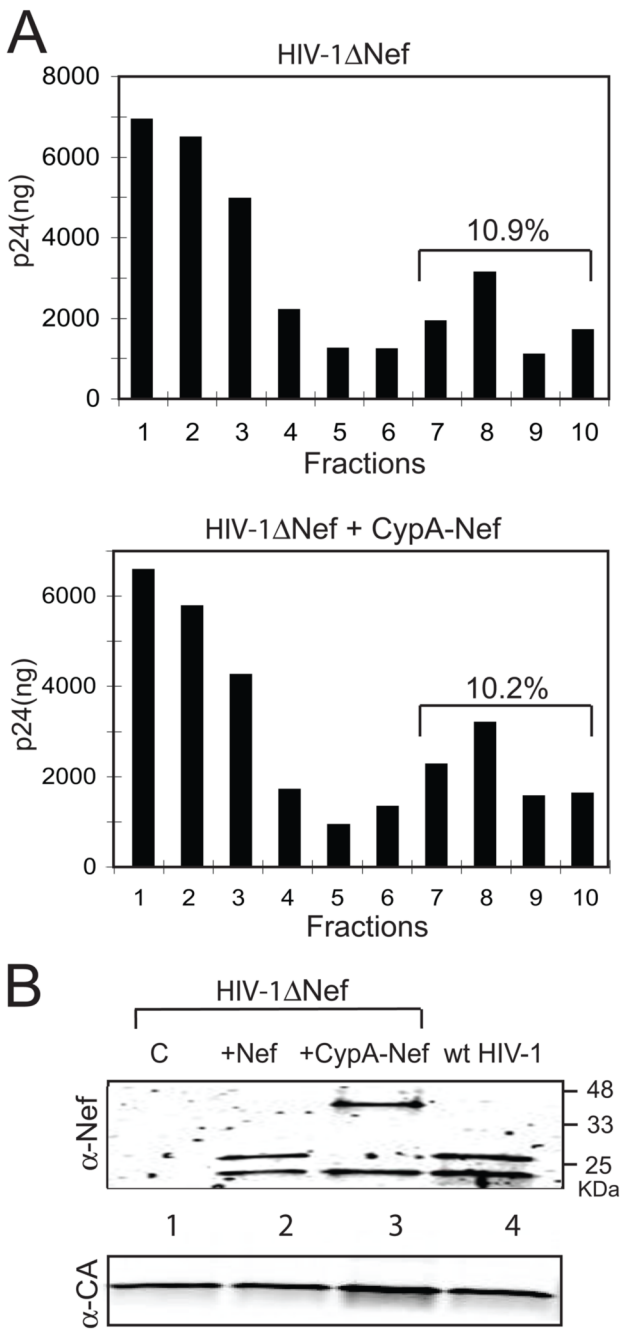

Fig. 3.

CypA-Nef copurifies with HIV-1 cores. Concentrated HIV-1 particles were sedimented through a layer containing 0.5% Triton X-100 into a linear sucrose density gradient. (A) Fractions were collected from the top and p24 levels were quantified by ELISA. Top panel: Nef-defective HIV-1; bottom panel: Nef-defective HIV-1 produced by contransfection with the CypA-Nef expression construct. (B) Immunoblot analysis of purified HIV-1 cores. Cores present in the peak fraction (fraction 8) from each gradient were pelleted and analyzed by immunoblotting using antibodies specific for Nef and CA. Lane 1: Nef-defective HIV-1 cores; lane 2: cores isolated from Nef-defective HIV-1 particles produced in cells expressing wild type Nef; lane 3: cores isolated from Nef-defective HIV-1 particles produced in cells expressing

CypA-Nef; lane 4: wild type HIV-1 cores. The upper and lower panels show the proteins detected with antibodies to Nef and CA, respectively.

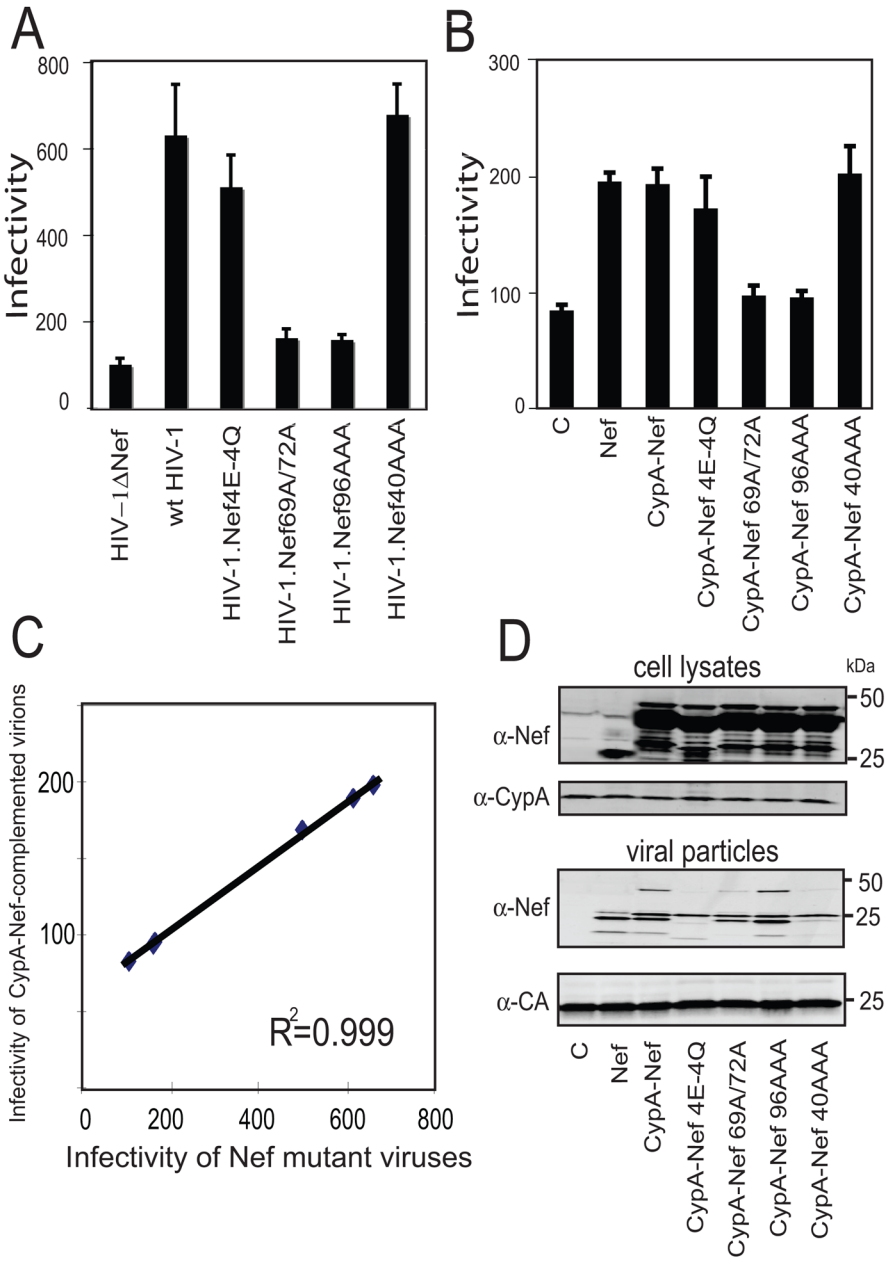

Fig. 4.

Point mutations in the Nef portion of CypA-Nef abolish infectivity enhancement. Viral supernatants were harvested and assayed for infectivity on P4 cells. The results shown are representative of 3 independent experiments. (A) Infectivity of HIV-1 mutants encoding substitutions in *nef*. (B) Infectivity of virions generated by cotransfection of the CypA-Nef mutants and *nef*-defective provirus. (C) Plot of infectivity of Nef mutant viruses (abscissa) vs. viruses complemented with the corresponding mutant CypA-Nef proteins (ordinate). (D) Immunoblot analysis of CypA-Nef expression in 293T cells (upper panel) and incorporation into Nef-defective HIV-1 particles (lower panel).

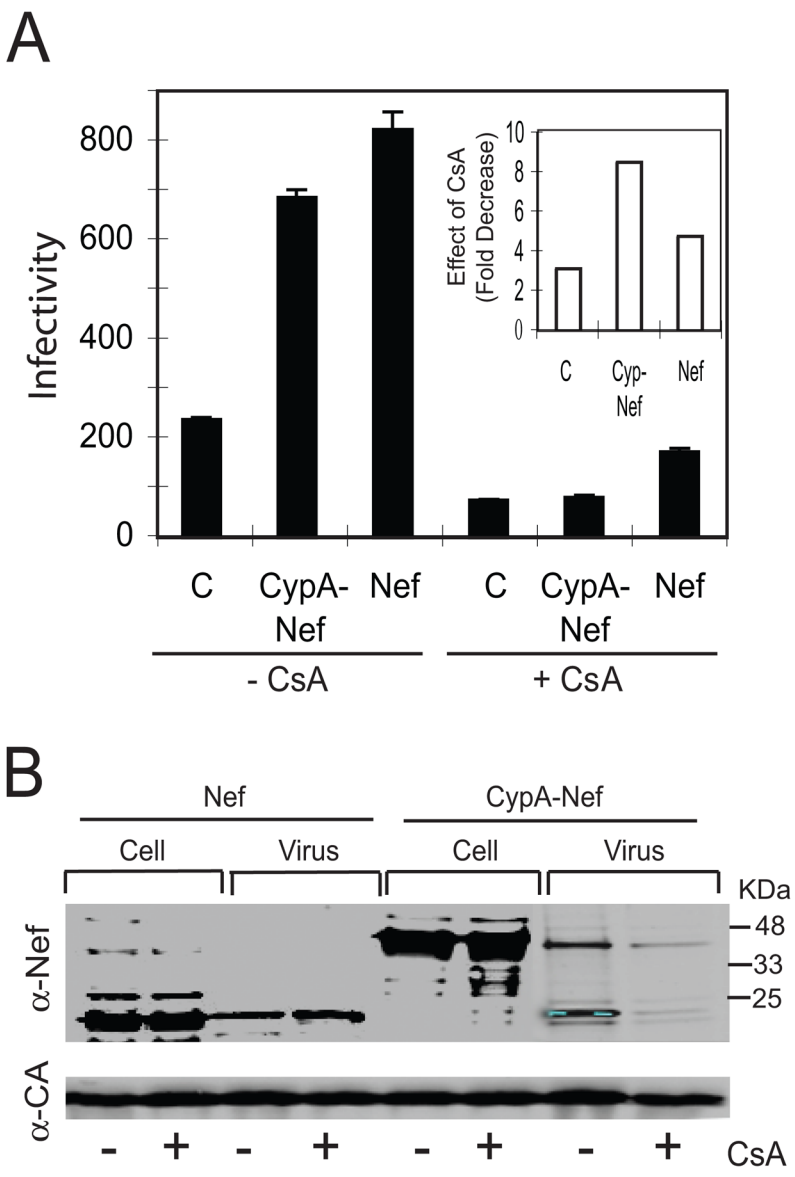

Fig. 5.

CsA inhibits HIV-1 incorporation of CypA-Nef and its ability to enhance viral infectivity. Viruses were produced in transfected cells cultured in the presence and absence of 5 μM CsA. (A) Infectivity of viruses on P4 indicator cells. Inset: fold inhibition of infectivity by CsA. Shown are the mean values of triplicate infections; results are representative of two independent experiments. (B) Immunoblot analysis of cell lysates and pelleted viruses using antibodies specific for Nef and CA.

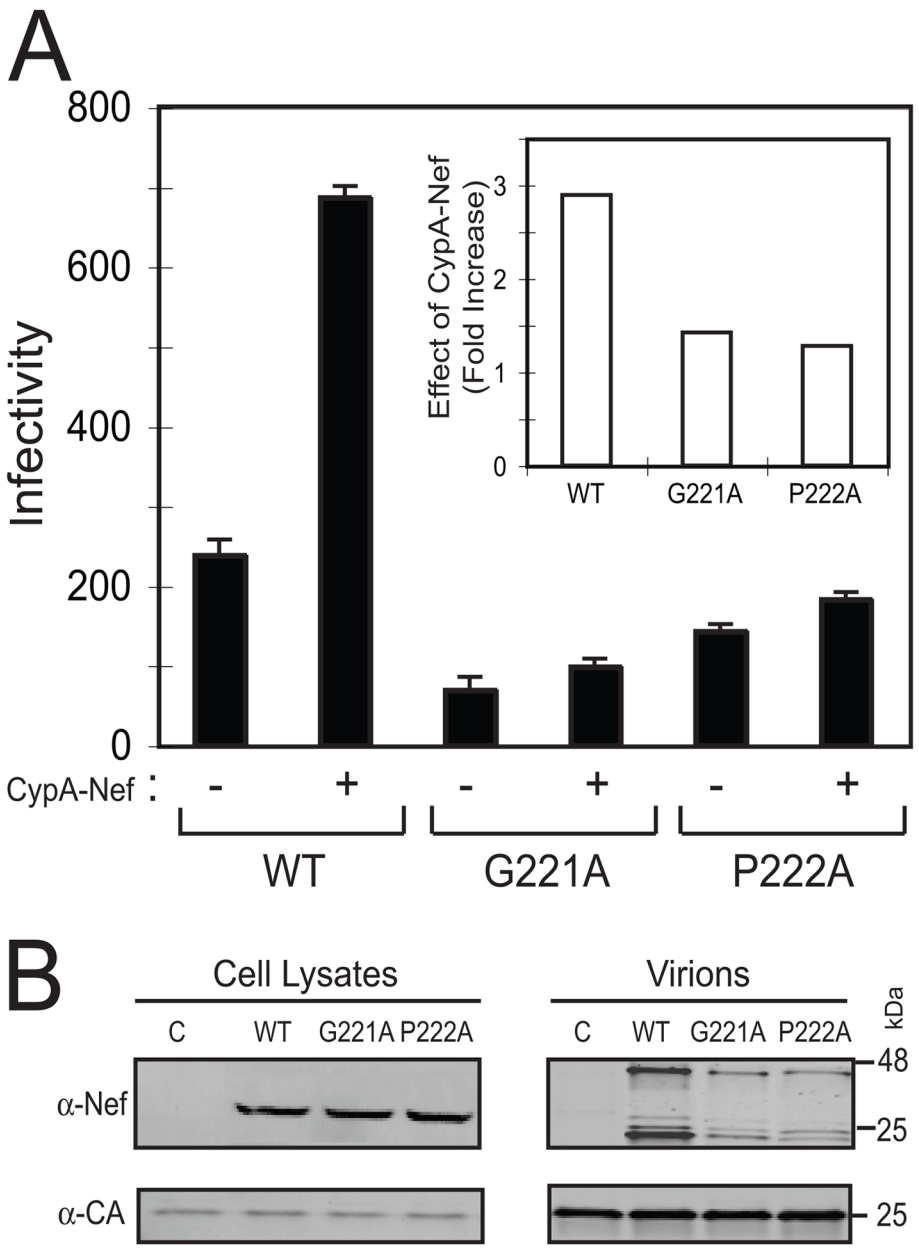

Fig. 6.

Mutations in the CypA-binding site in Gag inhibit HIV-1 incorporation of CypA-Nef and infectivity enhancement. Viruses were produced by cotransfection of Nef-defective proviruses containing the indicated mutations in Gag with plasmids encoding Nef or CypA-Nef. (A) Infectivity of the viruses. Inset: ratio of infectivity of viruses produced with and without coexpression of CypA-Nef. (B) Immunoblot analysis of cell lysates and pelleted viruses using antibodies specific for Nef and CA.

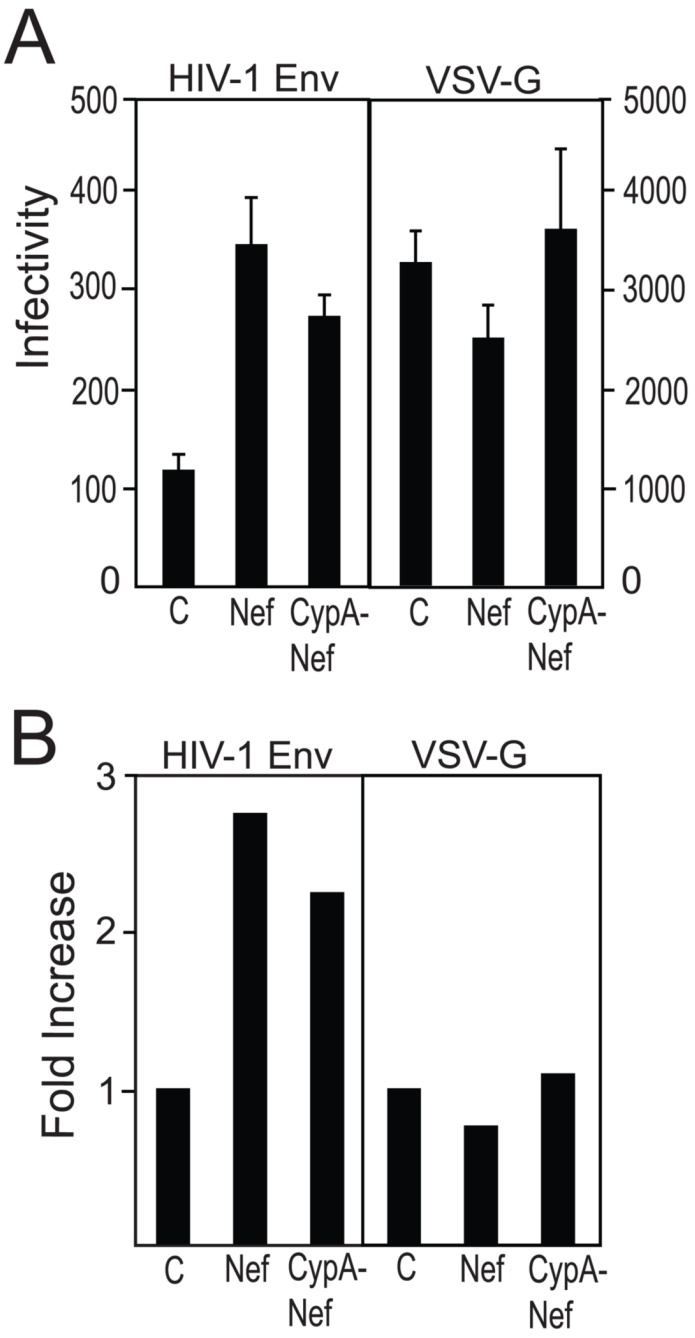

Fig. 7.

CypA-Nef enhances the infectivity of Nef-defective virions bearing native HIV-1 Env but not VSV-G. Viral particles were produced by transfecting 293T cells with R7.nef- provirus and Nef or CypA-Nef expression plasmids *in trans* (VC, vector control). For HIV-1(VSV-G) pseudotyped particles, the R7.nef-env- proviral construct was cotransfected with a VSV-G expression plasmid and the other indicated expression constructs. (A) Analysis of infectivity. Shown are the mean values of triplicate determinations, with error bars representing one standard deviation. The infectivity of each virus relative to its respective vector control is shown in panel (B).