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Downregulation of CD4 is required for maintenance of viral infectivity of HIV-1

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Abstract

Downregulation of virus receptors on the cell surface is considered to be important in preventing superinfection. HIV-1 encodes multiple gene products, Env, Vpu, and Nef, involved in downregulation of CD4, a major HIV-1 receptor. We found that simultaneous mutations in both *vpu* and *nef* severely impaired virus replication. We examined the involvement of CD4 downregulation mediated by Vpu and Nef in the modification of virus infectivity. The mutation in *vpu* increased CD4 incorporation into virions without affecting the Env content in it, inhibiting the attachment step of virions to the CD4-positive cell surface. Although a single mutation in *nef* suppresses virus infectivity via a CD4-independent mechanism, it could augment CD4 incorporation in virions in combination with a *vpu* mutation. These results indicated that CD4 downregulation was necessary for maintenance of Env function in the virion.

Introduction

The CD4 molecule is a major receptor for human immunodeficiency virus type 1 (HIV-1) entry (Klatzmann et al., 1984; Maddon et al., 1986) and plays a central role in acquired immune deficiency syndrome (AIDS) pathogenesis (Bour et al., 1995a). Decline of the CD4-positive cell population in the peripheral blood mononuclear cells (PBMCs) is a hallmark for the progression of AIDS. CD4 participates in the essential regulation in both development and function of the immune system. CD4 binds to the major histocompatibility complex type II (MHC-II) on antigen-presenting cells (APCs), which play a pivotal role for the antigen recognition machinery and activation of T cells (Weiss and Littman, 1994).

It has been reported that infection of HIV-1 in CD4-positive helper T cells, which are the major target for HIV-1 infection, induces downregulation of CD4 expression on the cell surface (Dalgleish et al., 1984; Klatzmann et al., 1984). Other enveloped viruses are also known to downregulate the receptor expression on infected cells, which is considered important in preventing superinfection (Delwart and Panganiban, 1989; Steck and Rubin, 1966). In most cases, the membrane-associated viral protein interacts with its receptor molecule on endoplasmic reticulum (ER) and/or cytoplasmic membrane, inducing downregulation of the receptor molecule from the cell surface. In HIV-1, it has been reported that the Env precursor, gPr160, binds to CD4 molecule in the ER and inhibits its transport to the cell surface (Cris et al., 1990; Jabbar and Nayak, 1990; Stevenson et al., 1988). In HIV-1 infections, many reports indicate that the viral regulatory gene products Vpu and Nef also participate in CD4 downregulation, in addition to Env (Chen et al., 1996; Garcia and Miller, 1991; Strebel et al., 1988). Recently Vpr has also been shown to suppress CD4 expression (Conti et al., 2000), even though there is a conflicting report (Arganaraz et al., 2002). Therefore, there appears to be a redundancy of gene function in CD4 downregulation. However, the mechanisms of these gene products in CD4 downregulation are functionally independent, and they act cooperatively to affect CD4 expression.

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Vpu and Env are translated from a bicistronic mRNA (Schwartz et al., 1990). On the contrary, most HIV-1 genes are expressed from monocistronic mRNAs, suggesting that a balanced expression of Vpu and Env is important for their functions. Vpu is a membrane-associated 16-kDa protein, which has an N-terminus embedded in the cytoplasmic membrane. A primary biological function associated with Vpu is an enhancement of virion release from HIV-1-infected cells (Strebel et al., 1988, 1989). Following this finding, it was reported that Vpu shortened the half-life of CD4 molecules, resulting in a reduction of cell-surface CD4 expression in HIV-1-infected cells (Willey, 1992a). Env precursor, gPr160, is involved in this activity; Vpu recognizes a gPr160/CD4 complex formed in the ER membrane (Bour et al., 1995b) and tags CD4 with ubiquitin ligase, βTrCP, directing proteasome-dependent degradation of CD4 (Margottin et al., 1998; Schubert et al., 1998).

Nef is a 27-kDa protein, of which the N-terminus is myristylated. It has been considered that Nef functions as a virulence factor for AIDS pathogenesis. Studies with long-term nonprogressors infected with HIV-1 reveal that deletions in *nef* are found in significant numbers in these populations (Kirchhoff et al., 1995; Mariani et al., 1996). In addition to that, simian immunodeficiency virus (SIV) Nef has been reported to play a crucial role in AIDS development in infected monkeys (Kestler et al., 1991). An experiment with transgenic mice harboring HIV-1 sequences suggested Nef was responsible for induction of AIDS-like symptom (Hanna et al., 1998). Although Nef function is dispensable in a virus replication assay using a T-cell line, it is required for enhancement of virus replication in PBMCs and macrophages (de Ronde et al., 1992; Miller et al., 1994; Spina et al., 1994; Terwilliger et al., 1991).

Nef has been reported to have multiple biological activities, one of which is downregulation of CD4 (Aiken et al., 1994; Garcia and Miller, 1991; Piguet et al., 1999b). Nef associates with CD4 molecules on the cell surface and directs clathrin-coated pit (CCP)-dependent endocytosis of CD4, resulting in lysosome-mediated CD4 degradation (Greenberg et al., 1997b; Mangasarian et al., 1997; Piguet et al., 1999a). For the sorting of CD4 by Nef, AP-2 has been reported to be involved (Greenberg et al., 1997a), but the details of the molecular mechanism remain to be elucidated. It has been reported that Nef can downregulate the MHC-I molecule through a distinct molecular pathway (Mangasarian et al., 1999; Riggs et al., 1999; Schwartz et al., 1996). Besides these activities, Nef has a function to enhance virus infectivity, by which virion infectivity is augmented by more than 10-fold in a single round of infection (Aiken and Trono, 1995; Cowers et al., 1994). The Nef function in virus infectivity is not dependent on CD4 expression in producer cells. The regulatory mechanism of virus infectivity by Nef remains to be further defined.

The fact that CD4 downregulation is mediated by multiple gene functions in HIV-1 implies that it plays an essential role in HIV-1 replication. Lundquist et al. (2002) reported that there was a clear correlation between the activity of Nef in CD4 downregulation and the replication capacity of the virus in CD4-positive T cells using a series of *nef* mutant viruses. However, the mechanism by which CD4 expression influenced virus infectivity was not fully documented. Elimination of the virus receptor on the cell surface makes it possible to avoid multiple superinfection events. Other reports showed that the accumulation of proviral DNA was caused by superinfection of HIV-1, which was toxic to the cells, indicating that it might be important for virus replication to inhibit superinfection (Bergeron and Sodroski, 1992; Pauza et al., 1990; Robunson and Zinkus, 1990; Somasundaran and Robinson, 1988). Additional reports stated that surface CD4 can be incorporated into virions, which prevents Env incorporation, resulting in impairment of virion infectivity (Coretes et al., 2002; Lama et al., 1999). It has also been reported that the cell surface CD4 binds to Env on virions, which inhibits virion release from the cell surface (Coretes et al., 2002; Ross et al., 1999). Although the effects of CD4 expression on virus infectivity have been shown in some experimental conditions, it remains unclear what role the modulation of CD4 expression plays in virus replication in vivo.

With *vpu* and *nef* mutant viruses, we examined the correlation between their effects on virus infectivity and on modulation of CD4 expression. These results indicated that Vpu affected the total amount of CD4 molecule, and the *vpu* mutation caused incorporation of CD4 into virions, resulting in impairment of virus infectivity. The amount of Env in virions was not affected by the *vpu* mutation, suggesting that the reduction of infectivity was due to the inhibition of attachment process caused by Env–CD4 association on the virion surface. Nef may function to eliminate cell surface CD4 expression, which is involved in the inhibition of viral superinfection.

Results

Vpu-modulated infectivity of virus produced from CD4-positive cells

Multiple gene products of HIV-1, Vpu, Nef, and Env, act cooperatively in downregulation of CD4, the major receptor for HIV-1 infection (Chen et al., 1996). Such functional redundancy for the CD4 downregulation implies that it plays an essential role in the virus life cycle, even though the biological significance remains to be elucidated. In order to investigate the contribution of CD4 downregulation to virus replication, the viruses harboring frame-shift mutations in *vpu* (Δ*vpu*), *nef* (Δ*nef*), and both *vpu* and *nef* (Δ*vpu*/Δ*nef*) were constructed from an HIV-1 infectious DNA clone, pNL432, and their infectivities were examined.

A model for replication capacity of each mutant virus, Δ*vpu*, Δ*nef*, or Δ*vpu*/Δ*nef*, is illustrated in Fig. 1A. Mutation in *nef* is expected to affect virus replication in T-cell

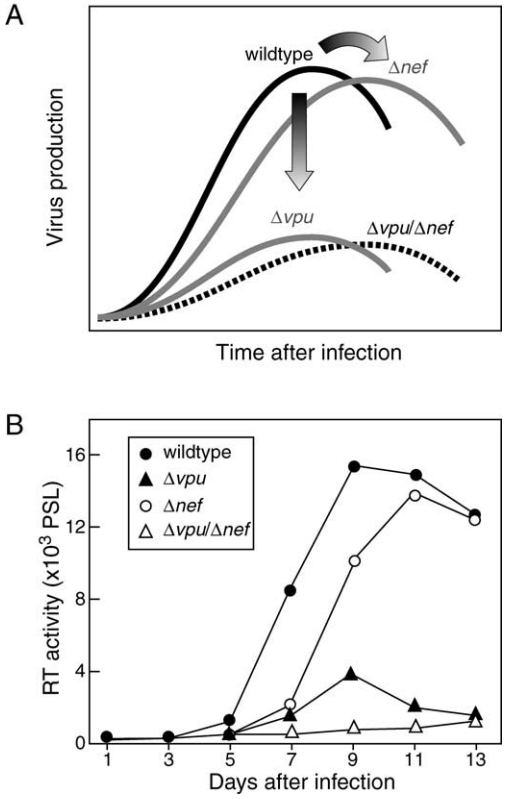

Fig. 1. Replication profiles of HIV-1 Δ*vpu*, Δ*nef*, and Δ*vpu*/Δ*nef* mutants. (A) A model of the replication profile of Δ*vpu*/Δ*nef* double mutants predicted from the data previously obtained with Δ*vpu* and Δ*nef* mutants (Adachi et al., 1991). (B) Growth profiles of the HIV-1 mutants. A3.01 cells were infected with each mutant (25 ng p24) on day 0 and the virus production in the culture supernatants at the indicated days after infection was measured by the RT assay. Three independent experiments were performed and the representative results are shown. The incorporation of [^{32}P]. TTP by RT activity was quantitated with a BAS-2000 analyzer (Fuji Film, Japan) and indicated by PSL unit.

lines only moderately. It has been reported that the infectivity of Δ*nef* virus is reduced as compared with wildtype, resulting in the moderate retardation of growth kinetics (Aiken and Trono, 1995; Cowers et al., 1994). The growth kinetics of Δ*vpu* virus is comparable to that of wildtype but the amount of virus particles released in culture supernatant is reduced. This is likely to be due to the function of Vpu in the enhancement of virus release from the surface of producer cells (Strebel et al., 1988). The infectivity of the released virions of Δ*vpu* virus has been reported to be comparable to that of wildtype (Sakai et al., 1995; Strebel et al., 1989). Assuming that the effects of Vpu and Nef are functionally independent, the growth profile of Δ*vpu*/Δ*nef* virus is expected as shown in Fig. 1A.

Growth potentials of the mutant viruses were examined in a T-cell line, A3.01 (Fig. 1B). Each virus having Δ*vpu* or Δ*nef* mutations showed a growth profile similar to that of the model illustrated in Fig. 1A. The double mutation in *vpu* and *nef* (Δ*vpu*/Δ*nef*) caused not only reduction of virion productivity but also severe retardation of growth kinetics. Similar results were obtained with the infection experiment with a different T-cell line, H9, and with PBMC (data not shown). Such a cooperative effect by Δ*vpu* and Δ*nef* on viral replication potential was previously suggested by Tokunaga et al. (1998), and it suggested that there was a functional linkage between Vpu and Nef functions.

Both Vpu and Nef are known to function in modulation of CD4 expression (Chen et al., 1996), suggesting that the phenotype observed with Δ*vpu*/Δ*nef* mutant was a consequence of downregulation of CD4 expression. To confirm the effect of CD4 expression on the virus replication, we examined the infectivity of virus derived from either CD4-positive or -negative cells using MAGI reporter cells (Fig. 2A). The mutant viruses were produced from either 293T cells, CD4-negative human kidney-derived cells, or 293T cells transfected with CD4 expression vector. The infectivity of wildtype virus was not affected by CD4 expression. Δ*Nef* virus showed moderately reduced infectivity as compared with that of wildtype. CD4 expression caused a slight reduction in infectivity of a Δ*nef* virus in a dose-dependent manner. The infectivity of Δ*vpu* virus obtained from CD4-negative cells was comparable to that of wildtype. It was significantly reduced, however, when CD4 was expressed in producer cells. The infectivity of the Δ*vpu*/Δ*nef* virus derived from CD4-positive cells appeared to be severely impaired. The result of a similar experiment with viruses derived from infected A3.01 cells resembled that of CD4-positive 293T cells (Fig. 2B), indicating that the regulatory effects by *vpu* and *vpu*/*nef* mutations on virus infectivity were dependent upon CD4 expression in producer cells.

Cell surface CD4 does not inhibit HIV-1 virion release

The result shown in Fig. 2A indicates that the defect of Δ*vpu* and Δ*vpu*/Δ*nef* viruses was located in the early phase of virus infection. It was still possible, however, that those mutations were also affected in the late phase, such as virion production. Ross et al., (1999) argued the effect of surface CD4 expression on virion release. They suggested the possibility that the surface CD4 could associate with gp120 on virions, causing trapping of virions on the cell surface. Both Vpu and Nef function in the CD4 downregulation, suggesting that the mutations in *vpu* and/or *nef* reduce the efficiency of virion release into the culture supernatant. By introducing CD4 expression plasmid and proviral DNA into 293T cells, we evaluated the virion release in culture supernatant (Fig. 3). It appeared that each mutation did not affect the efficiency of virion release from both CD4-negative and -positive 293T cells. The virus release was evaluated by measuring reverse transcriptase (RT) activity in the supernatant in this experiment, and we confirmed there was a linear correlation between RT activity and the amount of p24 with the mutant viruses used in this report (data not shown). Bour et al. (1999) reported that the expression of cell-surface CD4 interfered with Vpu function, resulting in inhibition of virion release. Because 293T cells were used in this report,

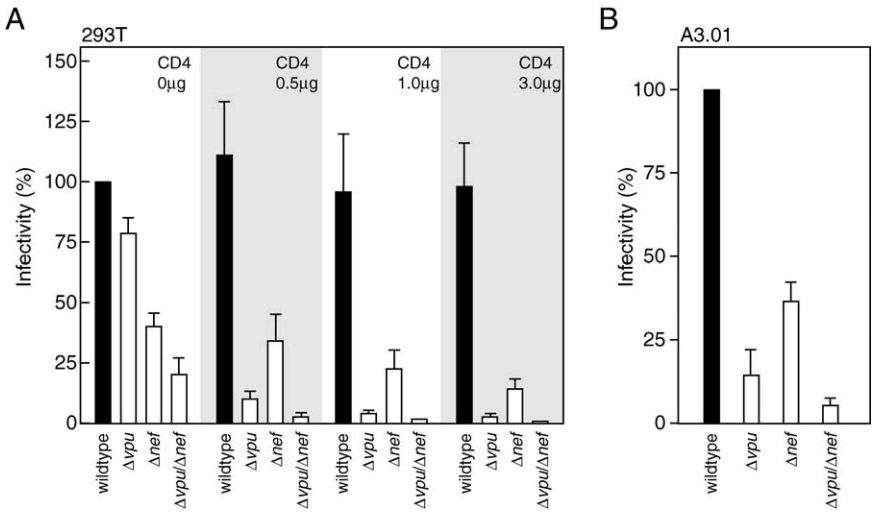

Fig. 2. The CD4-dependent effect of HIV-1 Δ*vpu*, Δ*nef*, and Δ*vpu*/Δ*nef* mutants on virion infectivity. (A) 293T cells were transfected with infectious HIV-1 DNA (5 μg) with or without a CD4 expression plasmid. The amounts of input CD4 expression plasmid are indicated in the figure. By using MAGI cells as an indicator, the infectivities of the viruses produced from transfected 293T cells were monitored. The amounts of input virus used in MAGI cell assays were adjusted by RT activity. The infectivity of wildtype virus obtained from CD4-negative 293T cells is normalized to 100% and the relative infectivity of the other viruses is shown. The results were obtained from at least three independent experiments, and the standard deviations are indicated with error bars. (B) The infectivity of the viruses derived from infected A3.01 cells were evaluated by the MAGI cell assay. Viruses were harvested on the day of maximal virus production. Equal amounts of virus, adjusted by RT activity, were used as input for the MAGI cell assay. The infectivity of wildtype virus is normalized to 100%. The results were obtained from at least three independent experiments, and the standard deviations are indicated with error bars.

in which Vpu-dependent enhancement of virion release was inactive, we cannot argue that there is an effect of interaction between Vpu and surface CD4. The results obtained here indicate that the remarkable impairment of infectivity observed with Δ*vpu*/Δ*nef* mutant is due to a defect in the early phase of infection.

A Vpu mutation causes CD4 incorporation into virions

The reduced infectivity observed with Δ*vpu* and Δ*vpu*/Δ*nef* viruses obtained from CD4-positive cells might be due to the modification in the composition of viral proteins in the virion. Lama et al. (1999) reported that mutations in *vpu* and/or *nef* abolished CD4 downregulation, resulting in loss of Env in virion. We examined whether such an effect was responsible for the reduced infectivity of the Δ*vpu* and Δ*vpu*/Δ*nef* viruses shown in Fig. 2A. In this case, CD4-negative 293T cells were used and the composition of viral proteins both in the virion and the cell extract appeared not to be affected by those mutations (Fig. 4A). By using CD4-expressing 293T cells as the virus producer, although some reduction of Env incorporation in virion was observed with each mutant, there was no significant difference among the mutants, suggesting that it was not responsible for the drastic decrease in infectivity caused by Δ*vpu* and Δ*vpu*/Δ*nef* mutations. Similar analyses were performed using infected A3.01 cells as the producer, and no significant difference was observed in viral protein composition either in the virion or in cell extracts between wildtype and mutant viruses (Fig. 4B). These results indicate that the reduction of infectivity found with Δ*vpu* and Δ*vpu*/Δ*nef* viruses from CD4-positive cells is not due to the consequence of the decreased Env incorporation.

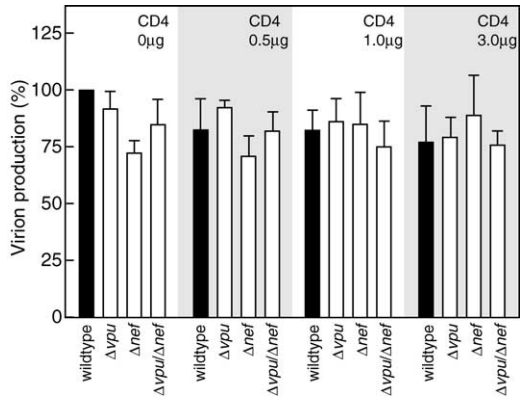

Fig. 3. The effect of CD4 expression on virion release. 293T cells were transfected with HIV-1 proviral DNA (5 μg) and the indicated amounts of CD4 expression plasmid. Virion production into culture medium was measured by the RT assay. The production of wildtype virus from CD4-negative cells is normalized to 100%. The results were obtained from at least three independent experiments, and the standard deviations are indicated with error bars.

We also examined the effects of *vpu* and/or *nef* mutations on the CD4 expression level in producer cells and virions (Fig. 4C). With CD4-expressing 293T cells, cell-associated CD4 was reduced with wildtype and Δ*nef* viruses, but not with Δ*vpu* or Δ*vpu*/Δ*nef* viruses, suggesting that the total amount of CD4 was mainly downregulated by Vpu function. CD4 molecules appeared to be significantly incorporated into Δ*vpu* virions, which were augmented by *vpu*/*nef* double mutation. CD4 incorporation into virions by *vpu* and

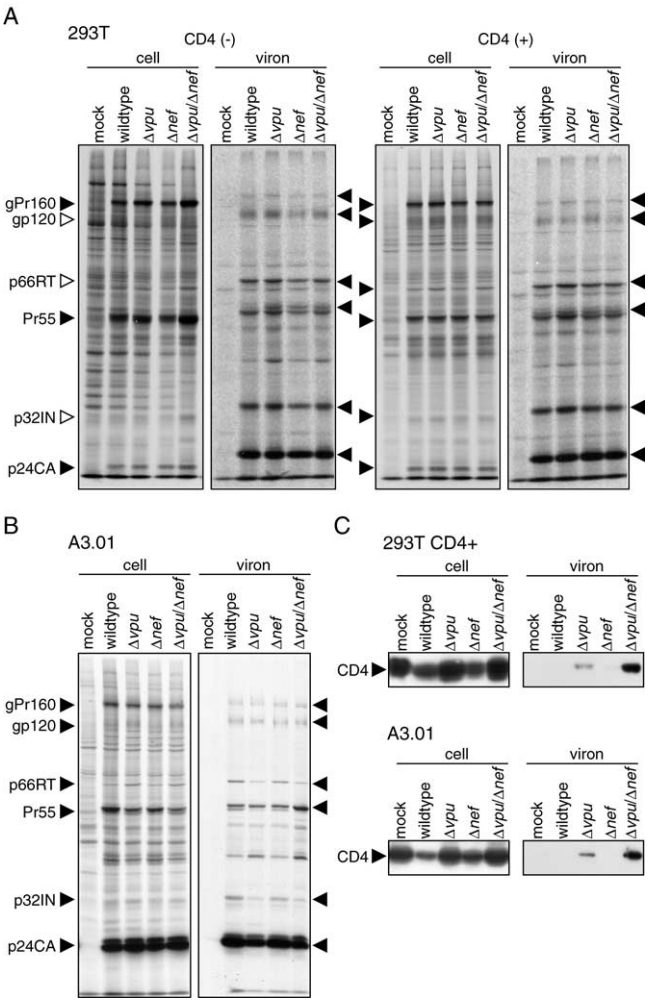

Fig. 4. Modification of the composition of viral proteins and CD4 by *vpu* and/or *nef* mutations. (A) Viral protein expression in the transfected cells and the produced virions were analyzed by immunoprecipitation with anti-HIV-1 serum. Infectious HIV-1 DNA (9 μg) was transfected into 293T cells with or without a CD4 expression plasmid (0.5 μg). Whole cell extracts and virion lysates were applied to a SDS–PAGE after adjusting the input amount with the total protein and amounts of p24, respectively. The positions of the major viral components are indicated by arrowheads. The viral proteins invisible in whole cell extract are indicated by open arrowheads. (B) An analysis similar to that shown in (A) was performed with A3.01 cells infected with HIV-1 mutants. A3.01 cells were infected and virus production and spread of the infected cell population were monitored each day after infection. The virion samples were harvested on the day of maximal virus production. The cell extracts were prepared when over 90% of cells became HIV-1 positive, which was estimated by immunostain of the cells with anti-HIV-1 serum. (C) CD4 expression was analyzed with the same samples used in (A) and (B). CD4 protein was detected by Western blot analysis with CD4 antiserum. Arrowheads indicate the position of CD4 protein.

vpu/*nef* mutants could be also observed by using A3.01 cells as the producer. These findings suggest that CD4 incorporation into virions contributes to the reduced infectivity of Δ*vpu* and Δ*vpu*/Δ*nef* viruses.

Coexpression of CD4 with Env on the virion surface may cause association between these molecules and interfere with the specific binding of Env to the cell surface CD4 receptor. In order to examine the inhibitory effect of virion-associated CD4 on virion attachment, a virus attachment assay was performed with mutant viruses produced by CD4-negative or -positive 293T cells (Fig. 5). With CD4-negative cells, the attachment efficiencies of all mutant viruses were comparable to those of wildtype. On the contrary, in a comparison of attachment efficiencies of the viruses derived from CD4-positive cells, *vpu* mutants were significantly reduced in efficiency. *Vpu*/*nef* double mutants more effectively abolished the attachment efficiency than single *vpu* mutants. This result correlates well with those shown in Figs. 1B and 2A. suggesting that the regulation of virus infectivity by Vpu function was dependent on CD4 expression in producer cells and that CD4 incorporation into virions had a negative effect on virion infectivity.

Nef functions in downregulation of surface CD4 molecule and plays an important role in inhibiting superinfection of HIV-1

There are many reports that describe CD4 downregulation by Nef. We found that Vpu function was important for the downregulation of the total amount of CD4 in A3.01 and CD4-expressing 293T cells and that Nef did not significantly affect CD4 expression (Fig. 4C). The *nef* mutation we used introduced a frameshift at the 35th amino acid position from its N-terminus, and we could not observe Nef protein expression with the Δ*nef* virus (Fig. 6A). Nef has been reported to induce internalization of surface CD4 through a CCP-dependent transport pathway, resulting in

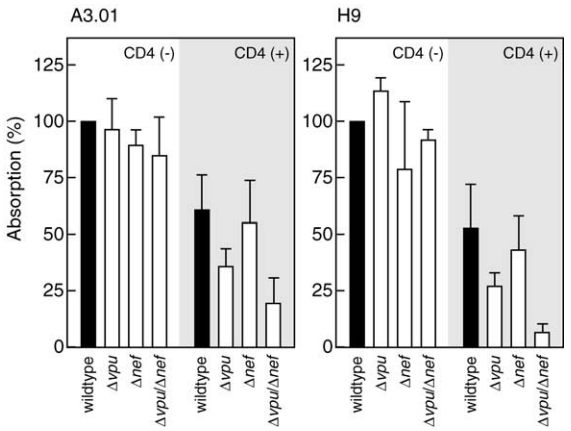

Fig. 5. Attachment efficiency of the virions produced from CD4-negative or CD4-positive cells. Virions were prepared from the transfected 293T cells as described in Fig. 2A. Virions corresponding to 100 ng of p24CA were incubated with A3.01 and H9 cells at 4°C for 1 h. After extensive washing, the cells were lysed and the cell extracts were applied to a p24 ELISA. The value obtained with wildtype virions derived from CD4-negative cells was normalized to 100%. The results were obtained from at least three independent experiments, and the standard deviations are indicated with error bars. The probability from Student's *t* test analysis for each data as compared to wildtype (CD4-) is lower than 0.05 ($P < 0.05$).

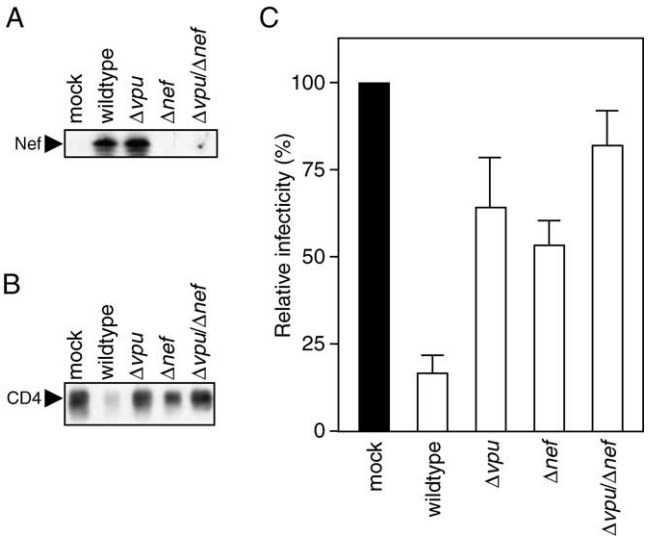

Fig. 6. Nef regulates virus superinfection through downregulation of surface CD4 molecules. (A) 293T cells were transfected with wildtype or mutant HIV-1 proviral DNA (9 μg), and the cell extracts were analyzed for Nef expression by Western blot with Nef antiserum. (B) 293T cells were transfected with HIV-1 proviral DNA (9 μg) with or without a CD4 expression plasmid (0.5 μg). Cell surface proteins were labeled by biotinylation and the whole cell extract was used in an anti-CD4 immunoprecipitation followed by anti-biotin Western blotting. (C) The superinfection assay was performed with the transfected 293T cells described in (B). An HIV-1-based reporter virus was introduced into the 293T cells and luciferase activity in the cells was analyzed 1 day after infection. The luciferase activity observed with mock-transfected 293T cells is normalized to 100%. The results were obtained from at least three independent experiments, and the standard deviations are indicated with error bars.

lysosome-dependent CD4 degradation (Greenberg et al., 1997b; Mangasarian et al., 1997); therefore, we investigated the surface CD4 expression on virus producer cells. Wildtype virus strongly suppressed CD4 expression on the cell surface (Fig. 6B). Δ*vpu*, Δ*nef*, and Δ*vpu*/Δ*nef* viruses, however, remarkably lost the effect on surface CD4 expression. This result indicated that Nef did not affect the total amount of CD4, but efficiently suppressed cell surface CD4 expression. Vpu downregulates surface CD4 through the degradation of intracellular CD4. Because the downregulation of surface CD4 is considered to contribute to the inhibition of superinfection, we examined the inhibitory effects on superinfection of Δ*vpu*, Δ*nef*, and Δ*vpu*/Δ*nef* mutant viruses. The efficiency of superinfection was evaluated by the infection of HIV-1-based reporter virus into 293T cells expressing both CD4 and the mutant virus. Although the expression of wildtype virus could effectively inhibit superinfection, both Δ*vpu* and Δ*nef* viruses significantly lost such an inhibitory effect (Fig. 6C). The cells infected with a Δ*vpu*/Δ*nef* double mutant virus appeared to allow superinfection to the degree of the control cells (mock, CD4-expressing 293T cells). This result indicated that both Vpu and Nef function in inhibition of superinfection and that Env does not play a major role in this function because Env expression was not affected by Δ*vpu*/Δ*nef* mutation.

Discussion

It has been reported that the elimination of virus receptor from cell surface is important not only for inhibition of superinfection but also for virus replication (Lundquist et al., 2002). In this report, we showed that the CD4 downregulation caused by HIV-1 infection is important for maintenance of virion infectivity. CD4 molecules were significantly incorporated when CD4 downregulation was hampered by *vpu* and/or *nef* mutations, resulting in reduced attachment efficiency of virions to target cells. Although Lama et al. (1999) reported that *vpu* and/or *nef* mutations abolished Env incorporation in exchange for CD4 incorporation in virions, we could not observe the effect on Env incorporation by the mutations. Because both they and we used the same cell line, 293T cell, for virus producer, the different observations could be due to the expression level of CD4 molecules in the producer cells, but careful comparison of the experimental conditions is required to explain this discrepancy. We analyzed the virion composition produced from a T-cell line, A3.01 cell, and the Env proteins appeared to be normally incorporated in virions of Δ*vpu* and Δ*vpu*/Δ*nef* mutants, suggesting that these mutations have a small effect on Env incorporation in physiological conditions. Similar results were obtained with another T-cell line, H9 (data not shown).

Vpu targets CD4 on the ER for proteasome-dependent degradation, which decreases the total amount of CD4 as shown in Fig. 4C (Bour et al., 1995b; Margottin et al., 1998; Schubert et al., 1998). Although the effect of Vpu on cell surface CD4 expression was comparable to that of Nef (Fig. 6B), the CD4 incorporation into the virion was specific to the Δ*vpu* virus. The result raised a possibility that the CD4 bound to gPr160 in the ER was transported and directly incorporated into virions in association with Env. An early report described that the association between CD4 and gPr160 in the ER attenuated transport of gPr160 to the cytoplasmic membrane and that Vpu rescued Env processing by diminishing CD4 through degradation (Willey et al., 1992b), however, our observation was in contrast to that. In CD4-expressing 293T cells, the *vpu* mutant did not affect the expression profile of Env in cells or the amount of matured gp120 in the virion, indicating that the association between CD4 and gPr160 did not affect the Env transport to the cell surface, its maturation, or its incorporation into the virion.

Although Vpu affects the total amount of CD4, Nef appears to target the CD4 molecule on the cell surface, which is consistent with previous reports. As we and other groups have reported, CD4 expression in producer cells does not contribute significantly to the Nef-mediated regulation of virion infectivity (Aiken and Trono, 1995; Miller et al., 1995). These results were, however, obtained with 293T cells; therefore, it is possible that Nef-mediated regulation of CD4 expression functions in enhancement of virus infectivity with PBMCs. As mentioned in the report by

Lundquist et al. (2002), the enhancement of virus infectivity by Nef was found to be more drastic with the viruses derived from PBL than with those derived from 293T and HeLa cells. It is necessary to investigate the biological significance of Nef-mediated CD4 downregulation in virus replication in PBMCs.

We found a severe reduction of infectivity by the simultaneous mutation of *vpu* and *nef* (Δ*vpu*/Δ*nef*). Although the reduction by the *vpu* mutation could be due to CD4 incorporation into virions, which by a *nef* mutation has been reported to be mostly independent of CD4 expression (Aiken and Trono, 1995; Miller et al., 1995). As shown in Fig. 2A; however, the infectivity of Δ*nef* virus was reduced with higher levels of CD4 expression, indicating that the downregulation of CD4 was partially involved in Nef-mediated promotion of virus infectivity. CD4-dependent regulation of virus infectivity by Nef is also supported by the fact that CD4 incorporation into virions is stimulated by Δ*vpu*/Δ*nef* to a larger extent than by a Δ*vpu* mutation. The regulation of virus infectivity by Nef through CD4 downregulation could be suppressed in the presence of Vpu and may become noticeable in the absence of Vpu. It might be also possible that CD4-independent enhancement of virus infectivity by Nef is augmented by the Δ*vpu* viruses which are defective for the attachment process.

A major purpose of Nef-mediated CD4 downregulation is likely to be the inhibition of HIV-1 superinfection, because Nef directs downregulation of cell-surface CD4, and that the Nef-mediated regulation of virus infectivity is mostly CD4-independent. It has been reported that superinfection of HIV-1 induces a cytotoxic effect, suggesting that the inhibition of superinfection is beneficial to efficient virus replication (Bergeron and Sodroski, 1992; Pauza et al., 1990; Robunson and Zinkus, 1990; Somasundaran and Robinson, 1988). However, this result was obtained by in vitro tissue culture studies, and therefore it needs to be investigated whether similar effects are observed under in vivo conditions. In infected individuals, superinfection may become permissive in latently infected cells, in which the expression of viral genes, including *vpu* and *nef*, are suppressed. In the multiply infected cells, recombination between viruses might produce an adapted virus under selective pressure from the host immune system, suggesting that superinfection may not be necessarily harmful for virus replication in vivo. Therefore, it is necessary to examine the significance of superinfection in in vivo studies (Le Guern and Levy, 1992; Robertson et al., 1995a, 1995b).

In this report, we described that CD4 downregulation caused by HIV-1 regulatory gene products is required for maintenance of virus infectivity, even though it remains unknown whether or not it is a major function of CD4 modulation in the virus life cycle. Although it is necessary to further investigate the molecular mechanism of Vpu- and Nef-mediated CD4 regulation and its contribution to virus replication, the results presented in this report raise the possibility that a mutant CD4 molecule resistant to Vpu- and Nef-mediated degradation would be a good candidate for anti-HIV reagents.

Materials and methods

Cell culture and DNA transfections

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS). The CD4-positive T-cell lines A3.01 (Folks et al., 1985) and H9 (Popovic et al., 1984) were maintained in RPMI 1640 medium containing 10% FBS. Hela–CD4–LTR–β-gal cells (MAGI cells: multinuclear activation of a galactosidase indicator) (Kimpton and Emerman, 1992) were propagated in DMEM supplemented with 10% FBS, 0.2 mg/ml G418 (Nacalai Tesque, Japan), and 0.1 mg/ml hygromycin B (Sigma–Aldrich Corp., St. Louis, MO). The calcium phosphate coprecipitation (Nishimura et al., 2000) was used for transfection. Cells (5×10^5) were seeded in a 6-cm dish 1 day prior to transfection. Plasmid and carrier DNA (total 10 μg) were incubated with 500 μl of Hepes-buffered saline (HBS) transfection buffer [140 mM NaCl, 0.75 mM Na_2HPO_4, 25 mM Hepes, 110 mM $CaCl_2$] (pH6.90) for 30 min at room temperature and then added to a culture dish. At 20 h after transfection, cells were washed once with phosphate-buffered saline (PBS) and fresh growth medium was added.

Reverse transcriptase (RT) assay

The RT assay was performed as described previously (Willey et al., 1988). Virus-containing culture medium was mixed with the RT assay cocktail [50 mM Tris · HCl (pH8.0), 75 mM KCl, 10 mM dithiothreitol (DTT), 4.95 mM $MgCl_2$, 10 μg/ml polyA, 5 μg/ml oligo dT, 0.05% (v/v) NP40, 0.4 MBq [α-^{32}P]TTP] and incubated for 1 h at 37°C. DE81 (Whatman International Ltd., Maidstone, England) was spotted with the reaction mixture and then washed with 2 × SSC [0.3 M NaCl, 30 mM sodium citrate (pH 7.0)]. Incorporated radioactivity was quantitated with a BAS2000 bioimaging analyzer (Fuji Film, Japan), by which the values were obtained as photo-stimulated luminescence (PSL) unit.

Virus replication assay

A3.01 cells (1×10^6 cells) were infected with the virus stock (25 ng p24) for 5 h in the presence of 8 μg/ml polybrene and then fresh growth medium (3.5ml) was added to the cells. The amounts of input virus were adjusted by RT activity. On the indicated days after infection, the culture supernatant was harvested and RT activity in the supernatant was quantified.

DNA constructs

An infectious proviral clone of HIV-1, pNL432 (wild-type) (Adachi et al., 1986), and its mutants, designated pNL-Ss (Δ*vpu* mutant), pNL-Xh (Δ*nef* mutant), and pNL-Kp (Δ*env* mutant), have been previously described (Adachi et al., 1991). pNL-SsXh (Δ*vpu*/Δ*nef* double mutant) was constructed from pNL-Ss by introducing frameshift mutation in the *nef* coding region (*XhoI* site). The pNL–Δrev–luc was constructed from pNL432 which contains a point mutation at the rev ATG initiation codon (ATG was substituted with ATC) and an insertion of the luciferase coding gene (derived from pGL3-basic, BD Biosciences Clontech, Palo Alto, CA) in the nef region (*Xho*I site). The rev expression plasmid, pCMV–F-Rev, was described previously (Ono et al., 2000). CD4 expression plasmid, pCMV4–CD4 was constructed by inserting the CD4 coding DNA derived from T4–pMV7 into the pCMV4 expression plasmid (Del Vecchio et al., 1992). T4–pMV7 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Richard Axel (Maddon et al., 1986).

MAGI cell assay

MAGI cells were infected with virus stock (adjusted with the RT activity) in the presence of 8 μg/ml polybrene, and the β-galactosidase activity in the cells was monitored at 72 h after infection by the Luminescent β-gal Reporter System 3 (BD Biosciences Clontech, Palo Alto, CA).

Preparation of cell extracts and virions

The cells were lysed in RIPA buffer [150 mM NaCl, 50mM Tris · HCl (pH8.0), 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM DTT, 16 μg/ml benzamidine HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM PMSF]. Virion-containing culture medium was loaded onto 20%/65% discontinuous sucrose gradient and centrifuged at 30 krpm for 2 h (RPS-40T rotor, Hitachi Koki Co., Ltd., Japan). The interphase of the sucrose gradient was collected and the virions were pelleted by ultracentrifugation (30 krpm, 2 h, 4°C). The virion pellet was resuspended in RIPA buffer.

Western blot analysis

Cell extracts and virion samples were loaded on 10% SDS–polyacrylamide gel electrophoresis (PAGE). The proteins were transferred onto Hybond–PVDF membrane (Amersham Biosciences Corp., Piscataway, NJ) and Nef and CD4 proteins were detected with HIV-1 Nef antiserum (Shugars et al., 1993) and CD4 antiserum (T4-4) (Deen et al., 1988; Willey et al., 1992b) (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), respectively. For visualization, a chemiluminescent detection reagent (Lumi-Light Western Blotting Substrate, Roche Diagnostics GmbH, Mannheim, Germany) was used.

Metabolic labeling and immunoprecipitation

At 48 h posttransfection of the proviral DNA clones into 293T cells, the cells were labeled for 12 h with Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham Biosciences Corp., Piscataway, NJ). The cell extracts and virus particles were prepared in RIPA buffer. Viral proteins were precipitated with anti-HIV-1 serum (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, HIV-1 neutralizing serum(1)) (Vujcic and Quinnan, 1995) plus protein G–agarose (Invitrogen Corp., Carlsbad, CA) and separated through 10% SDS–PAGE. The gel image was obtained by exposure to BioMax MS films (Eastman Kodak Company, Rochester, NY).

Cell surface protein analysis

The cells were washed three times with PBS, biotinylated at 4°C for 30 min with 0.5 mg/ml of NHS-SS-Biotin (Pierce, Rockford, IL) in PBS, and lysed with RIPA buffer. The cell extracts were precipitated with CD4 antiserum (T4-4) and protein G–agarose and then separated on 10% SDS–PAGE. The biotinylated CD4 was detected by Western blot analysis with anti-biotin monoclonal antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA).

Virion attachment assay

For the attachment assay, A3.01 and H9 cells (1×10^6 cells) were incubated with viruses (100 ng p24). After 1 h of incubation at 4°C, unbound virions were removed by washing with ice-cold PBS containing 1% FBS. The cells were lysed in a lysis buffer [0.5% (v/v) NP-40, 10 mM NaCl, 10 mM Tris · HCl (pH7.5)], and then the amount of cell-associated p24 in the cell extract was determined with a p24 ELISA (ZeptoMetrix Corp., Buffalo, NY).

Superinfection assay

293T cells were transfected with HIV-1 proviral DNA (9 μg), pCMV4–CD4 (0.5 μg), and pGreenLantern-1 (Invitrogen Corp., Carlsbad, CA). The transfection efficiency was monitored by the expression of green fluorescence protein and the samples over 90% transfection efficiency were used for the assay. Two days after transfection, the cells were infected with an HIV-based reporter virus in the presence of polybrene (8 mg/ml). At 24 h postinfection, the cells were harvested and analyzed for luciferase expression by the Luciferase Assay System (Promega Corp., Madison, WI). Reporter virus was obtained from 293T cells transfected with pNL–Δrev–luc (9.5 μg) and pCMV–F-rev (0.5 μg).

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