Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner

Juan Lama*†, Aram Mangasarian†‡ and Didier Trono‡

Background: Human immunodeficiency virus-1 (HIV-1) infection decreases the cell-surface expression of its cellular receptor, CD4, through the combined actions of Nef, Env and Vpu. Such functional convergence strongly suggests that CD4 downregulation is critical for optimal viral replication, yet the significance of this phenomenon has so far remained a puzzle.

Results: We show that high levels of CD4 on the surface of HIV-infected cells induce a dramatic reduction in the infectivity of released virions by the sequestering of the viral envelope by CD4. CD4 is able to accumulate in viral particles while at the same time blocking incorporation of Env into the virion. Nef and Vpu, through their ability to downregulate CD4, counteract this effect.

Conclusions: The CD4-mediated 'envelope interference' described here probably explains the plurality of mechanisms developed by HIV to downregulate the cell-surface expression of its receptor.

Addresses: *La Jolla Institute for Allergy and Immunology, Molecular Immunology Division, 10355 Science Center Drive, San Diego, California 92121, USA. ‡Department of Genetics and Microbiology, CMU, 9 Avenue de Champel, CH-1211 Geneva 4, Switzerland.

Correspondence: Didier Trono
E-mail: didier.trono@medecine.unige.ch

†J.L. and A.M. contributed equally to this work.

Received: 15 April 1999
Revised: 17 May 1999
Accepted: 18 May 1999

Published: 3 June 1999

Current Biology 1999, 9:622–631
http://biomednet.com/elecref/0960982200900622

 ISSN 0960-9822

Background

The recognition of the cell-surface glycoprotein CD4 by the envelope of human immunodeficiency virus (HIV) initiates a series of events that culminates in the fusion of the viral and cellular membranes and in the delivery of the inner components of the virion into the cytoplasm of target cells [1–5]. Subsequently, CD4 expression on the surface of HIV-1-infected cells is downregulated by the combined actions of three viral proteins: Nef, envelope (Env) and Vpu [2,4,6–10]. Of these, Nef acts the most rapidly, both because it is an early viral gene product [11,12] and because it triggers the accelerated internalization of CD4 molecules that have already reached the cell surface [13–15]. In contrast, Env and Vpu are produced from a bicistronic late viral mRNA [16] and affect newly synthesized CD4 molecules. Env forms a complex with CD4 in the endoplasmic reticulum (ER) [17,18] and Vpu triggers the degradation of receptor molecules thus retained in this early compartment [10]. Although mechanistically distinct, the effects of Nef, Env and Vpu are additive, ensuring the almost complete elimination of CD4 from the surface of cells productively infected with HIV-1 [13,19].

Despite this good understanding of the mechanisms of HIV-induced CD4 downregulation, the functional significance of this event is not understood, although a few hypotheses have been formulated. Eliminating the main viral receptor from the surface of HIV-infected cells might avoid multiple superinfection events that could lead to premature cell death [20]. Alternatively, by analogy with the action of influenza virus neuraminidase, CD4 downregulation could prevent the trapping and aggregation of outgoing virions on the surface of producer cells [21–23]. Finally, CD4 regulation could alter T-cell activation pathways to favor viral gene expression, for instance by increasing the intracellular levels of free Lck tyrosine kinase, as observed in other systems [24]. However, none of these models is yet supported by concrete experimental evidence.

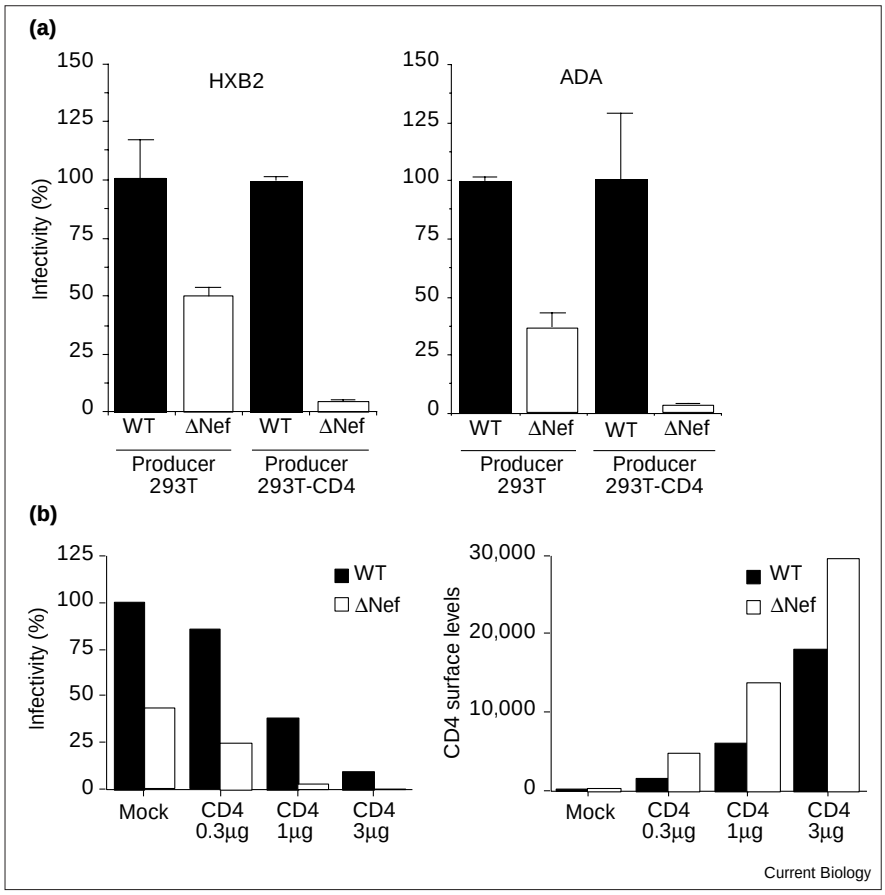
Difficulty in maintaining a productive HIV infection in cell lines expressing high levels of CD4 has been reported [25], but the viral countermeasures were not investigated and the mechanism of the inhibition was not elucidated. Here we reveal an important role for CD4 downregulation in HIV infection, by showing that this phenomenon is crucial for preserving the full infectivity of HIV-1 virions.

Results

Dose-dependent inhibition of HIV-1 infectivity by CD4

An effect of CD4 on HIV-1 infectivity was first assessed using a luciferase-expressing HIV-based retroviral vector system. Vector particles were produced by transfecting either 293T or 293T-CD4 cells with a mixture of three DNAs as described previously [26–28]: an HIV-derived packaging plasmid, an Env expression construct, and the retroviral vector DNA itself. Wild-type and *nef*-defective versions of the packaging construct, as well as HIV-1 Env derived from either the T-cell tropic (T-tropic) HXB2 or

Figure 1



CD4-mediated inhibition of HIV-1 infectivity. (a) Luciferase-expressing HIV-1 vector particles coated with either the T-tropic HXB2 (left) or the M-tropic ADA (right) envelope were produced by transient transfection of 293T or 293T-CD4 cells with a wild-type (WT) or a *nef*-defective packaging construct. Relative infectivity was determined by measuring luciferase production in HOS-CD4-CCR5 target cells, normalizing for p24 antigen content of the inoculum. CD4 expression did not affect the efficiency of particle release. Results, expressed as a percentage of the infectivity of wild-type particles produced from CD4-negative 293T cells, represent the mean ± standard error (SEM) determination from three experiments. (b) Wild-type or *nef*-defective HXB2-Env-coated vector particles were produced from 293T cells transfected with increasing amounts of a CD4-expressing plasmid. The amounts of luciferase activity induced in HOS-CD4-CXCR4 target cells by the resulting virus are shown in the left panel. The levels of CD4 in 293T cells, determined using flow cytometry at the time of virus harvest, are shown in the right panel. CD4 levels are given in arbitrary units, calculated by multiplying the percentage of CD4-positive cells by the median of the fluorescence signal as previously described [66].

the macrophage-tropic (M-tropic) ADA isolates, were compared. Infectivity was tested by measuring the luciferase activity induced in HOS-CD4-CCR5 cells exposed to the virions, and normalizing for the p24 capsid antigen content of the inoculum (Figure 1a). These cells express both the CXCR4 and the CCR5 HIV-1 co-receptors, and are thus susceptible to both T- and M-tropic HIV-1 strains [29]. CD4 expression had no effect on the efficiency of particle release and did not affect the infectivity of wild-type virions. It significantly further decreased the infectivity of particles produced from the *nef*-deleted packaging construct, however; from two- to threefold for virus released by 293T cells to more than 20-fold for virions generated in 293T-CD4 cells.

The fact that CD4 affected the infectivity of *nef*-defective (ΔNef) but not wild-type virus suggested that the inhibition might be proportional to the cell-surface levels of CD4. To test this, luciferase-expressing vector particles coated with the T-tropic HXB2 Env were generated in 293T cells transfected with increasing amounts of a CD4-expressing vector. The vector particles were then used to infect HOS-CD4-CXCR4 cells, which overexpress CXCR4. Infectivity was scored by measuring luciferase activity in these targets, normalizing for the amount of p24 in the inoculum. In addition, CD4 levels on the surface of producer cells were examined using flow cytometry (Figure 1b). Whereas small amounts of CD4 (following transfection of 0.3 μg CD4-expressing DNA) only slightly reduced the infectivity of wild-type virions, higher levels had a dramatic effect. For instance, wild-type vector particles produced in cells transfected with 3 μg CD4 DNA were more than 10 times less infectious that those produced in CD4-negative cells. The greater sensitivity of *nef*-defective viruses was confirmed in this experimental setting: transfection of 1 μg CD4 plasmid caused a twofold reduction in the infectivity of wild-type particles but a 10-fold reduction in that of *nef*-defective virions. In both cases, a strict correlation was observed between the surface levels of CD4 in virus-producing cells and the degree of CD4-mediated inhibition. Experiments with HIV-1 proviral DNA transfected in the presence of increasing amounts of CD4 expression vector gave similar results (data not shown). It should be noted that, in this system, CD4 did not affect the efficiency of virion production (data not shown).

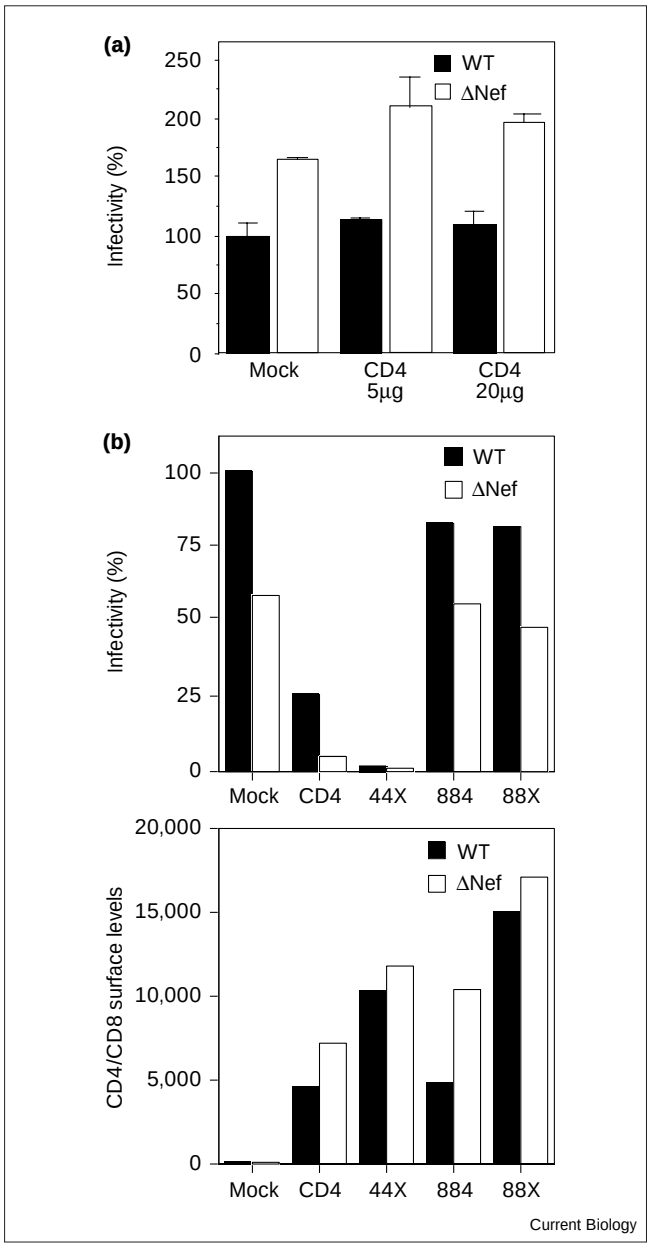


Figure 2

CD4-mediated inhibition of HIV-1 infectivity requires CD4–Env binding. (a) Relative infectivity of wild-type (WT) and *nef*-defective, luciferase-expressing, vector particles pseudotyped with the G protein of VSV and produced from 293T cells either mock-transfected (Mock) or transfected with increasing amounts of a CD4-expressing plasmid, using HOS-CD4-CCR5 cells as targets. Results are the means from two experiments each performed in duplicate. Data are presented as percentage of the infectivity of wild-type particles produced in the absence of CD4. (b) The same experimental system was used to test the inhibitory effect of co-transfection with 1 μg of expression vectors expressing various CD4 and CD8 derivatives. The three-letter code denotes the source of the various domains of these molecules (see text). The top panel shows the relative infectivity of particles on HOS-CD4-CXCR4 cells (with wild-type virions produced in the absence of inhibitor serving as reference). None of the CD4 and CD8 derivatives affected the efficiency of particle release (data not shown). The bottom panel shows cell-surface expression of CD4/CD8 derivatives, estimated using flow cytometry with antibodies specific for CD4 (that is, CD4 and 44X) or CD8 (that is, 884 and 88X). Results are representative of two independent experiments.

The inhibitory effect of CD4 requires an interaction with Env

A probable mechanism for the observed inhibition of HIV-1 infectivity is that CD4 interferes with Env function. To test this hypothesis, retroviral vector particles pseudotyped with the G protein of vesicular stomatitis virus (VSV) were produced in 293T cells transfected with increasing amounts of a CD4-expressing plasmid, and their infectivity was analyzed using HOS-CD4-CCR5 cells as targets (Figure 2a). As described previously [28,30], the absence of Nef did not impair the infectivity of the VSV G-pseudotyped HIV-1 virions but instead slightly stimulated it. Moreover, CD4 had no effect in this setting, even when expressed at levels higher than those required for almost complete inhibition of vector particles coated with HIV-1 Env. The CD4-mediated inhibition thus seems to require that viruses be coated with the HIV-1 envelope.

CD4 binds to the gp120 surface moiety of Env through its extracellular region [31], whereas the CD4 cytoplasmic tail, which associates with the protein tyrosine kinase $p56^{lck}$ (Lck), is the target of Nef and Vpu [13,32–38]. A CD4 derivative (44X) lacking the cytoplasmic domain is expressed at high levels on the cell surface and is insensitive to Nef [13,39]. As one would predict, this CD4 variant strongly inhibited the infectivity of both wild-type and ΔNef vector particles (Figure 2b). Reciprocally, a CD8/CD4 chimera (884) that contained the extracellular and transmembrane domains of CD8 fused to the cytoplasmic tail of CD4 did not significantly reduce the infectivity of HIV-based particles, even though this fusion protein was expressed at high levels on the cell surface. As expected, a truncated CD8 variant lacking a cytoplasmic tail (88X) behaved in a similar way. The extracellular domain of CD4 is therefore both necessary and sufficient for interference with HIV-1 infectivity, at least if it is part of an integral transmembrane protein. Furthermore, the rescuing effect of Nef apparently depends on the conservation of CD4 determinants that allow for downregulation by this viral protein. In support of this model, a CD4 variant mutated in a dileucine motif critical for Nef responsiveness [13] was equally active on wild-type and ΔNef virions (data not shown).

Nef and Vpu counteract the CD4-mediated inhibition of HIV-1 infectivity

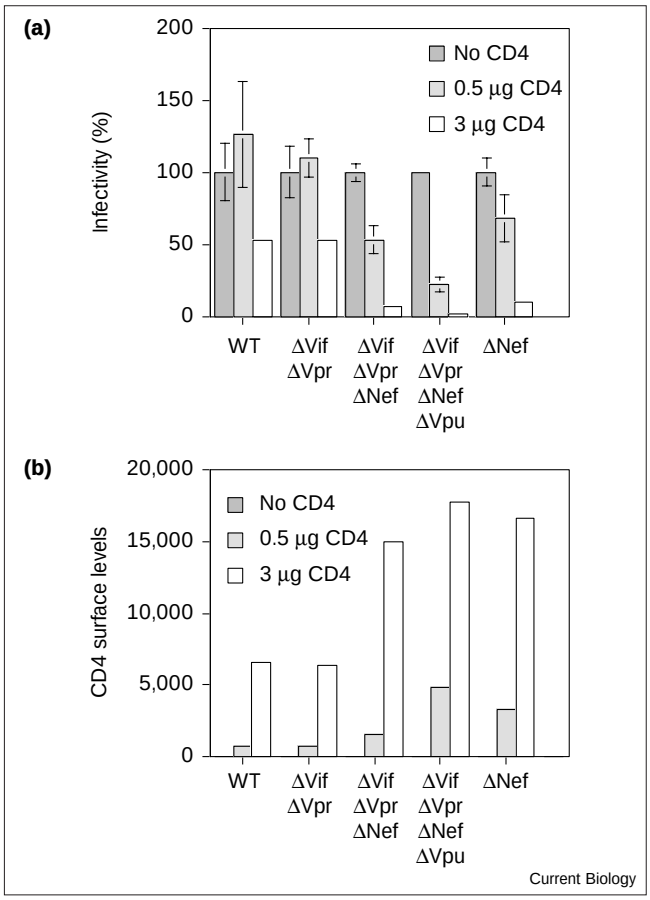
Our results indicate that the inhibition of HIV-1 infectivity by CD4 is proportional to the level of CD4 at the cell surface, possibly explaining how Nef prevents this inhibition. To confirm this and to evaluate whether Vpu, another downregulator of CD4, has a similar positive influence on

HIV-1 infectivity, we compared the CD4 sensitivities of vector particles generated from several singly and multiply deleted HIV-1 packaging constructs and coated with the HXB2 envelope (Figure 3). In the absence of CD4 expression, all vector particles exhibited comparable transduction efficiencies. In the presence of CD4, the efficiency of virion release was unaffected, irrespective of the packaging construct (data not shown). Furthermore, particles with defective virion infectivity factor (*vif*) or viral protein R (*vpr*) genes were as infectious as wild-type particles. In contrast, virions generated from packaging constructs additionally mutated in *nef*, or in *nef* and *vpu*, were impaired to extents proportional to the levels of CD4 on the surface of the producer cells. CD4 levels were highest on cells transfected with the quadruply defective packaging plasmid, and the infectivity of the virions produced in this case was the lowest. Nef and Vpu thus seem to exert additive influences to counteract the CD4-mediated inhibition of HIV-1 infectivity, most probably through their ability to induce CD4 downregulation. Because the transducing genome encoding luciferase does not encode any proteins necessary for its packaging, these experiments show that the accessory proteins Nef and Vpu work in *trans* to complement infectivity.

To confirm and extend the results obtained with the retroviral vectors, we tested the CD4 sensitivity of the following HIV-1 mutants: ΔNef, ΔVpu, ΔNef/ΔVpu, and Nef_{WL58AA}. This last mutation is of interest because it targets the CD4-binding pocket of Nef, almost completely inhibiting CD4 downregulation, while leaving much of the Nef-mediated infectivity enhancement intact if virion production occurs in a CD4-negative setting [40]. Transfected proviral DNAs were all derived from the previously described R9 clone, which expresses all HIV-1 accessory genes. When produced in the absence of CD4, both the *nef*-deleted virus and the double mutant deleted in *nef* and *vpu* were found to be approximately 15% as infectious as wild type, whereas the *vpu*-deleted and the Nef_{WL58AA} viruses retained roughly 60 and 40% of initial infectivity respectively (Figure 4a, top).

As in the vector system, the ability to resist the presence of CD4 in the producer cells correlated with the ability to downregulate CD4 in the viral producer cells (Figure 4a, bottom and Figure 4b). The mutant that experienced the most severe decline in infectivity relative to initial levels was the ΔNef/ΔVpu double mutant (96%), followed by ΔNef (93%), Nef_{WL58AA} (89%), and finally ΔVpu (77%) (Figure 4a, bottom). In contrast, the wild-type virus lost only 27% of its original infectivity when CD4 was present in the viral producer cells. The fact that the Nef_{WL58AA} and ΔNef HIV-1 derivatives responded to the introduction of CD4 with relatively similar large losses in infectivity suggests that CD4 downregulation is a significant component of the ability of Nef to enhance infectivity. The Nef_{WL58AA} mutant retained its higher absolute level of infectivity relative to ΔNef, confirming that two distinct effects contribute to Nef-mediated enhancement of HIV-1 infectivity: the downregulation of CD4, and the previously described stimulation of proviral DNA synthesis [41–43].

Figure 3

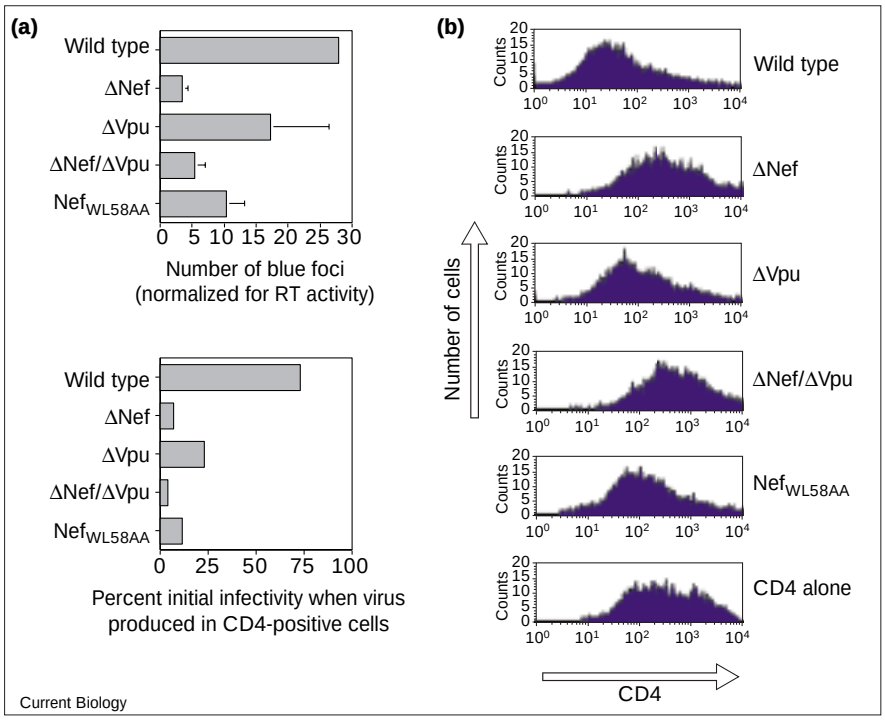


Nef and *Vpu* counteract the inhibitory effect of CD4. (a) Relative infectivity of retroviral particles coated with the HXB2 envelope and produced from packaging constructs either wild-type or mutated in various HIV-1 genes, in the presence or absence of CD4. For each type of vector, transducing ability in the absence of CD4 constituted the 100% reference value. Results are from two experiments each performed in duplicate ± SEM. (b) Cell-surface levels of CD4 in the 293T producer cells from a representative experiment, determined by flow cytometry. See text for description of mutants.

High cell-surface CD4 expression alters the Env content of HIV-1 virions

One possible explanation for the above observations is that CD4 sequesters the HIV-1 envelope. To test this possibility, we examined the protein content of virions produced in the presence and absence of CD4. This analysis revealed that CD4-mediated inhibition of viral infectivity probably has two components. First, there is an almost complete, CD4-dependent block to the proper incorporation of Env in viruses harboring deletions in *nef*

Figure 4



HIV-1 *nef* and *vpu* mutant viruses are more sensitive to CD4 expression in producer cells. (a) Relative infectivity of HIV-1 viral particles produced by transient transfection of 293T cells (top). Cells were transfected with 20 μg proviral DNA for viral production. Supernatants were filtered through 0.45 μm filters and normalized for reverse transcriptase (RT) activity before addition to target P4-2 cells, which harbor the HIV-LTR-driven β-galactosidase gene. The bottom panel shows the percentage of initial infectivity retained when proviral DNA was co-transfected with 1 μg CD4 expression vector. Results for each condition are from at least three separate transfection/infection series. (b) Cell-surface levels of CD4 on viral producer cells in a representative experiment following co-transfection of 1 μg CD4 expression vector with proviral DNA. See text for descriptions of mutants.

or *vpu*; second, high levels of CD4 are present in the *nef*- and *vpu*-defective virions (Figure 5a).

The Env content of the virions was measured by western blotting with antibodies against both the gp41 transmembrane moiety and the gp120 surface moiety of the envelope as we were concerned that the presence of CD4 on the particles might cause trapping of gp120 molecules shed from the cell surface. When released from CD4-expressing cells, viruses mutated in *nef* and/or *vpu* contained almost no gp41, and instead carried large amounts of CD4. In the absence of Vpu, however, the particles still had some gp120, whether *nef* was functional or not. Examination of cell-surface expression of Env in cells producing the various viruses solved this puzzle (Figure 5b). Env was almost 20 times more abundant on the surface of cells transfected with the ΔVpu proviral construct.

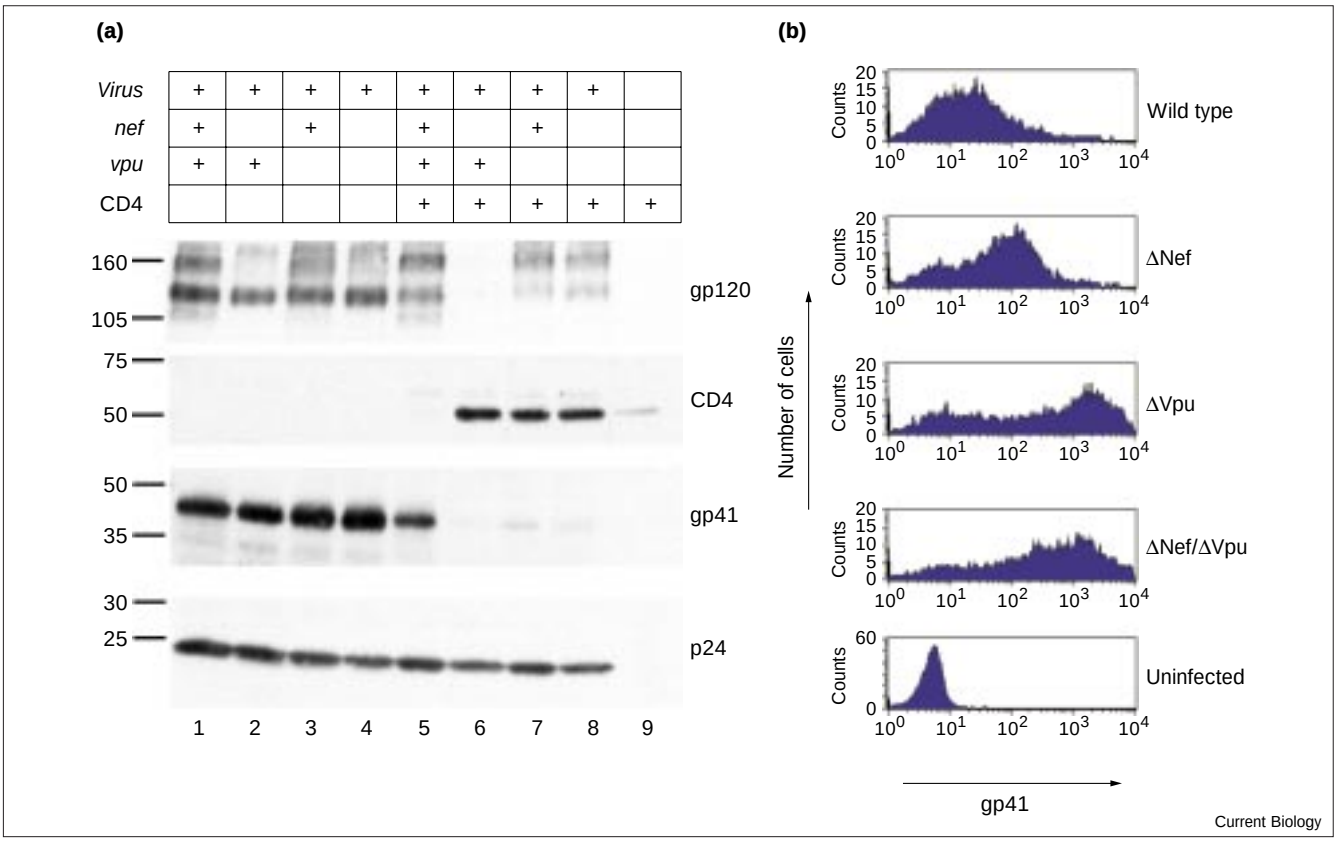
We attribute this effect to the fact that the *vpu* mutation used in these experiments is an inactivation of the *vpu* start codon. Because Vpu and Env are translated from the same bicistronic mRNA, the removal of the *vpu* initiator, located upstream, greatly facilitates Env synthesis. Higher levels of gp120 are shed into the supernatant and subsequently bind CD4 molecules carried by the virions. The increase in Env synthesis is reflected not only in its cell-surface expression, but also in the slightly increased levels of gp41 found in purified virions, for example when comparing the ΔNef and ΔNef/ΔVpu viruses in the presence of CD4. The high levels of Env on the surface of ΔVpu-producing cells contributed little to HIV infectivity, however, emphasizing the efficacy of the CD4-mediated block to Env incorporation into the virion. Interestingly, the cell-surface expression of Env was also increased in cells releasing ΔNef HIV-1, compared with wild-type infected cells. This effect was much less pronounced than in ΔVpu-producing cells, however. As expected, we found that the CD4-mediated block to Env incorporation correlated with a decreased efficiency of viral entry into target cells, as measured by p24 uptake (data not shown).

Together with the functional analyses, these data indicate that CD4 inhibits HIV-1 infectivity by blocking the efficient incorporation of the viral envelope into the virion; the envelope, therefore, cannot act to mediate viral entry into cells. Our study also demonstrates that Nef and Vpu have evolved to counteract this inhibitory effect by downregulating the cell-surface expression of CD4.

Discussion

Cells infected with a retrovirus become resistant to reinfection by a virus bearing the same envelope glycoprotein [44,45]. This phenomenon is known as receptor interference, and for simple retroviruses such as the Rous sarcoma virus (RSV) or the murine leukemia virus (MLV) it reflects solely the action of Env [46]. Receptor downregulation is more complex in the case of HIV; Nef and Vpu

Figure 5

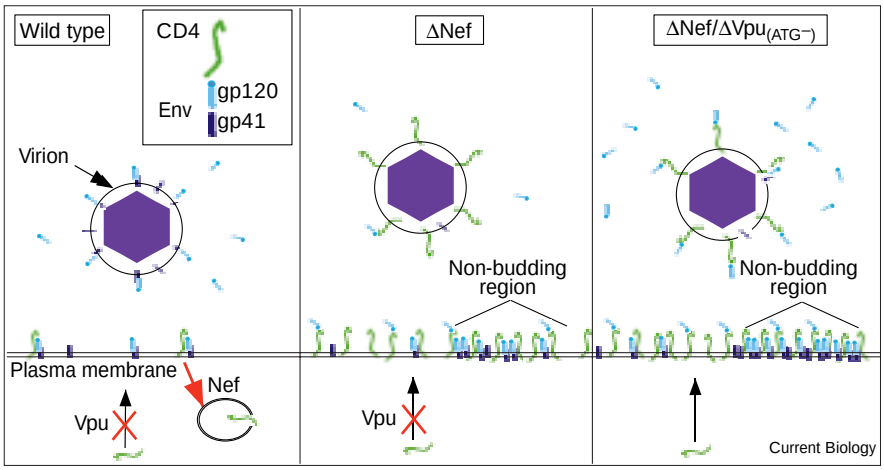


CD4 alters the protein content of HIV-1 virions. (a) Lanes 1–8 show western blots of virions purified by ultracentrifugation of supernatant from cultures of 293T cells two days after transfection with the various proviral constructs derived from the R9 wild-type clone (see text), which contains all HIV-1 accessory genes. Loading of samples was normalized according to reverse transcriptase activity. As a control, the supernatant of cells transfected with the CD4 expression vector only were processed in parallel (lane 9). As this sample contained no reverse transcriptase activity, we loaded a volume corresponding to the maximum volume loaded in the series of viral samples. A + sign in the rows above the western blots indicates, from top to bottom, that: proviral DNA was transfected into the viral producer cells (Virus); the proviral genome contained *nef*; the proviral genome contained *vpu*; and 1 μg of CD4 expression vector was cotransfected. The numbers at the left indicate molecular mass markers in kDa. (b) Infected CEM–GFP cells were analyzed by FACS for cell-surface expression of gp41, the transmembrane moiety of Env, using the T32 monoclonal antibody. Infected cells were gated by taking advantage of the HIV-LTR driven green fluorescent protein (GFP) cDNA present in these cells.

add their influence to that of the viral envelope to eliminate CD4 almost completely from the surface of productively infected cells. This functional redundancy was unexplained, even taking into account the higher cell-surface levels of CD4 compared with those of other known retrovirus receptors [47]. The work reported here suggests a solution to this puzzle, by showing that the combined actions of Nef and Vpu can prevent the CD4-mediated inhibition of virion infectivity that is observed when high levels of CD4 are present on the surface of HIV-1-infected cells. Inhibition by CD4 reflects a sequestration of the viral envelope by this cellular receptor, which results in a block of Env particle incorporation. We propose calling this effect 'envelope interference', by analogy with receptor interference, which it mirrors in some aspects (Figure 6).

The results reported here were in part obtained in transfected epithelial cells because this system best allowed examination of the relative effects of Nef, Vpu and CD4 in single and synchronous rounds of viral production. It has been noted previously, however, that the ability of a human T-lymphoid cell line to support and maintain a productive infection was inversely proportional to its surface levels of CD4 [25]. Furthermore, we had reported that the defective phenotype of a virus expressing a Nef variant unable to downregulate CD4 (Nef_{WL58AA}) was more pronounced, compared with wild type, when it was released from CD4-positive CEM T-lymphoid cells than when it was produced by CD4-negative 293 cells [40]. The present study provides the functional and biochemical data that explain these two observations.

Figure 6



Model of CD4-mediated inhibition of HIV-1 infectivity. In the wild-type virus infection (left panel), both Nef and Vpu assist in downregulating CD4, resulting in its near-complete elimination from the surface of the infected cell. Levels of Env on the cell surface are not very high, but incorporation into the virion is efficient. In the case of ΔNef virus (middle panel), CD4 levels are markedly increased on the surface of the virus producer cell. This has two effects: first, Env is sequestered on the cell surface, possibly in a non-budding region of the cell membrane, and is therefore not efficiently incorporated into the virion; second, the viral particle harbors CD4 on its surface. With a virus lacking both Nef and Vpu (right panel), the situation is similar except that, because of the absence of Vpu, CD4 levels are even higher on the cell surface, and because of the deletion of the *vpu* ATG in the bicistronic *vpu/env* mRNA, there is much more Env on the plasma membrane. This results in increased quantities of gp120 being shed in the extracellular space, with some of it binding to CD4 on the virus surface. Despite the greater quantity of Env protein on the cell when *vpu* is mutated, CD4 is able to block incorporation of almost all of this protein into the virus particle.

The HIV-1 envelope glycoprotein is synthesized as a 160 kDa precursor in the ER, where it undergoes several modifications including disulfide bonding, folding, N-linked glycosylation and oligomerization [48,49]. One demonstrated role of Vpu is to induce the degradation of CD4 molecules complexed with Env in the ER, and thereby to liberate the viral envelope [10]. Once transported to the Golgi apparatus, Env is cleaved into its gp120 surface and gp41 transmembrane moieties, and is further modified by alteration of its sugar residues before migrating to the cell surface. Steady-state levels of Env are then downregulated in at least two ways. First, a significant fraction of gp120 molecules is shed into the extracellular milieu [49]. Second, the glycoprotein also undergoes internalization owing to the presence of a tyrosine-based endocytosis signal in the cytoplasmic domain of gp41 [50,51]. Interestingly we found that levels of Env increased on the surface of viral producer cells in the absence of Nef. This effect was independent of the presence of CD4, as we noted a similar phenomenon in T lymphocytes and CD4$^-$ epithelial cells (data not shown). A negative effect of Nef on the cell-surface expression of the HIV-1 envelope has been reported previously [52]. In this study, it was also indicated that Nef does not affect the biosynthesis and maturation of the viral glycoprotein, nor the shedding of its gp120 moiety into the extracellular space. Our data are at odds with the previous claim that Nef-induced downregulation of cell-surface Env can be abrogated by mutations suppressing the CD4-binding ability of Env [52], as we observe this effect in a CD4-negative setting. The origin of this discrepancy is unclear, although in that other study a vaccinia-based expression system was used, virions were not examined, and CD4-negative producer cells were not tested. The results presented here suggest that the Nef-mediated downregulation of Env does not simply result from the internalization of CD4–Env complexes, and raise the possibility that Nef might influence the intracellular trafficking of Env itself, a point worth further investigation.

The HIV-1 envelope is normally recruited into virions through specific interactions between the cytoplasmic tail of gp41 and residues in the viral matrix protein (MA) [53–55]. It is noteworthy that CD4 can block this process without affecting the efficiency of viral budding. This suggests that Env has a much higher affinity for CD4 than for MA, or that MA cannot recognize Env within the context of CD4–Env complexes. This latter defect could result either from masking of critical Env determinants or from a redistribution of the viral glycoprotein away from the *gag* gene products when it is associated with CD4. One possibility is that Env is perhaps redirected to a 'non-budding' region of the plasma membrane by CD4. Lck, which associates with the cytoplasmic tail of CD4, is known to reside in structures known as rafts or detergent-insoluble glycolipid-enriched membrane microdomains (DIGs) [56]. Rafts are detergent-resistant regions of the membrane where the activation machinery of T cells is concentrated [57,58]. It would be interesting to determine whether CD4 also accumulates in rafts, even in the absence of Lck, and whether it can drag Env into these structures. It might also be instructive to ask whether the virus buds from regions of the plasma membrane that do or do not correspond to rafts. The efficient recruitment of CD4 into virions strongly suggests that the receptor might inhibit HIV-1 infectivity not only by decreasing the efficiency of Env incorporation into the virus particle, but also by saturating

the CD4-binding sites of virus-associated envelope molecules (Figure 6).

Nef can stimulate the infectivity of HIV-1 virions independently of its ability to downregulate CD4 [41,42,59,60]. This positive effect of Nef is exerted at a post-uptake level but is inapparent when virions enter cells by receptor-mediated endocytosis, and correlates with a stimulation of proviral DNA synthesis which probably reflects a stabilization of the viral reverse transcription complex [30,41–43]. Here we show that Nef-induced CD4 downregulation also participates in promoting HIV-1 infectivity, as *nef*-defective mutant viruses, as well as an HIV-1 derivative expressing a CD4 downregulation mutant of *nef*, are significantly more sensitive to CD4 than their wild-type counterparts.

Conclusions

We reveal here that high levels of CD4 on the surface of an HIV-producing cell can block viral infectivity by interfering with incorporation of the envelope into the virion. Nef and Vpu, two accessory gene products of HIV-1 that downregulate CD4, counteract this inhibition. This suggests that blocking of Nef- and Vpu-mediated CD4 downregulation could have a profound impact on the spread of HIV-1 *in vivo*. The remarkable progress made in understanding the molecular mechanisms of action of these two viral proteins could be exploited by developing antiviral strategies that target their function.

Materials and methods

Plasmids

Expression of CD4 and CD4/CD8 chimeras was achieved with the cytomegalovirus (CMV)-based mammalian expression vector pCMX [61]. Plasmids used to generate HIV-1-derived vector particles carrying a luciferase reporter transgene have been described [26–28]. These particles were either pseudotyped with the G protein of VSV (VSV-G) or coated with the HIV-1 envelope glycoprotein. The T-tropic HXB2-derived HIV-1 envelope was produced from plasmid ΔGPVV-R7-dhfr, a variant of the previously described R7-dhfr construct [62] in which the sequences encoding Gag, Pol, Vpr, Vif and Nef have been inactivated. The M-tropic ADA-derived HIV-1 envelope expression vector was obtained from D. Littman [29]. HIV-1 virions were made using the previously described R9 construct, in which all HIV-1 accessory genes are present [40]. All mutations were made in this context. ΔNefR9 and Nef_{WL58AA}R9 have been described previously [40]. The *vpu* deletion mutants were constructed by a PCR mutagenesis strategy which results in the substitution of the first two codons of *vpu*: 5′-ATGCCA-3′ with 5′-ACTAGT-3′, destroying the initiation codon and introducing a *Spe*I site for verifying the presence of the mutation.

Cells

293T cells, a derivative of the human kidney fibroblastic 293 cell line that stably expresses the simian virus 40 large T antigen (a gift from G. Nolan), were used for production of HIV-1 particles. Some of the experiments were carried out with a 293T subclone, 293T-CD4, that stably expresses human CD4. HIV-1 infectivity assays were carried out in $CD4^+$ HeLa-derived P4 cells, which contain the β-galactosidase gene under the control of the HIV promoter, allowing the easy scoring of infected cells by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining [63]. Transduction efficiency of HIV-derived luciferase vectors was tested in HOS-CD4-CXCR4 or HOS-CD4-CCR5, which are human osteosarcoma cells modified to express CD4 and either the T-tropic CXCR4 or the M-tropic CCR5 co-receptors [29]. HOS-CD4-CCR5 expresses low levels of CXCR4 and can be infected by either T- or M-tropic HIV-1 isolates. Both cell lines were a gift from N. Landau, obtained through the National Institutes of Health AIDS Research and Reference Reagent Program. CEM-GFP cells (a gift from A. Gervaix) are a T-lymphocytic cell line with a stably integrated green fluorescent protein (GFP) expression cassette driven by the HIV-1 long terminal repeat (LTR) [64]. As a result, these cells inducibly express GFP in response to the presence of Tat, the HIV-1 transcriptional transactivator. GFP-positive cells are thus considered infected, and can be gated separately by fluorescence-activated cell sorter (FACS) analysis. All cells were grown in Dulbecco's modified Eagle's medium (DMEM), except for CEM-GFP cells, which were grown in RPMI, and supplemented with 10% fetal calf serum (FCS) with addition of 1 μg/ml puromycin (Sigma) for the HOS-derived lines.

Transfections, virus preparations and infections.

293T cells were transfected by the calcium phosphate method [39]. HIV-based particles pseudotyped with either HIV-1 gp120 or VSV-G proteins were harvested after 48 h and filtered through 0.45 μm nitrocellulose membranes. Samples containing vector particles were analyzed for p24 antigen content by an enzyme-linked immunoabsorbent assay (DuPont). Samples containing HIV were normalized by the reverse transcription (RT) assay (described below).

To examine virion-associated proteins, particles were pelleted at 26,000 rpm in an SW28 rotor (Beckman) for 1.5 h at 4°C. The liquid was aspirated, and the walls of the tube wiped before resuspending the pellet in 150 μl phosphate-buffered saline (PBS) for 10 min at room temperature. Samples were stored at 4°C until RT determination was performed (approximately 5 h later). Samples were then diluted with PBS to give equal concentrations of RT activity, and then denatured by the addition of 1/6 volume of 6× sample buffer (500 mM Tris–HCl, 12% SDS, 60% glycerol, 0.01% bromophenol blue, 20% β-mercaptoethanol), followed by heating at 96°C for 5 min.

To determine the infectivity of HIV-1 particles, 5×10^4 P4 cells per well (seeded in 24-well trays) were infected in 1 ml of DMEM supplemented with 10% FCS for 24 h with amounts of virions corresponding to 1/1, 1/5, 1/25, and 1/125 dilutions of the least concentrated supernatant after normalization for RT activity. The medium was changed 24 h after infection, and cells were incubated for an additional 24 h at 37°C. Cells were then fixed and stained with X-gal. The numbers of blue foci were counted under the light microscope.

For preparation of HIV-based retroviral particles coated with either VSV-G or gp120, a previously described procedure was followed [26–28] with some modifications. 293T cells (1.5×10^6) were seeded the day before transfection. Transfection was carried out with a mixture of four plasmids: either 2.5 μg pMDG (encoding VSV-G) or 3.5 μg HIV-1 *env* expression vector, 7.5 μg packaging construct (encoding various HIV-1 proteins but not Env), 10 μg of the transfer vector pHR′-CMVluc (expressing luciferase under the control of the immediate early CMV promoter) and various amounts of a pCMX-based CD4 expression vector. After 2 days, supernatants were filtered through 0.4 μm nitrocellulose membranes. To determine the infectivity of these particles, 1×10^5 HOS-derived cells (seeded in six-well trays 24 h before infection) were exposed to 50 μl supernatant (containing approximately 25 ng p24) in 2 ml DMEM supplemented with 10% FCS and 8 μg/ml polybrene. Twelve hours after infection the medium was replaced. Two days later the cells were washed twice with TBS buffer (50 mM Tris-HCl pH 7.8, 130 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$) and lysed in 200 μl of the same buffer supplemented with 0.5% Nonidet P-40 (NP-40). After clarification by centrifugation (14,000 rpm for 5 min in a microfuge), lysates were assayed for luciferase activity.

The reverse transcriptase cocktail consisted of: 60 mM Tris-Cl pH 7.8, 75 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM EDTA, and 0.16 μg/ml poly(A):oligo(dT). Just before use, 4 μl 1 M DTT, 20–40 μl [^{3}H]TTP

(1 mCi/ml) were added to each ml of cocktail. A sample of 20 µl of 1:10 dilution of detergent-inactivated virus (0.5% Triton X-100) was added to 40 µl of the cocktail, and incubated for 2 h at 37°C. For each determination, duplicate dilutions and RT reactions were performed. The entire reaction was spotted on 2.3 cm Whatman DE81 circles that were washed three times for 5 min in 2× SSC, and once in 95% ethanol, before scintillation counting.

Protein analyses and antibodies

The cellular and viral levels of specific proteins were examined by western blotting with the following antibodies: for HIV-1 gp120 a mixture of three mouse monoclonal antibodies from the MRC AIDS repository was used: 301 (at 1:500),.323 (at 1:1250) and 332 (at 1:1250). For HIV-1 gp41 western and FACS analysis the supernatant of the T32 hybridoma cell line, a gift from Patricia Earl, was used [65]. T32 cells were grown in HyQ-CCM1 (Hyclone B5500-L) with 50 units/ml human IL-6. A monoclonal antibody from the NIH AIDS repository was used to probe p24 capsid proteins; for CD4, rabbit antiserum obtained from the NIH AIDS Research and Reference Reagent Program (AR 806) was used. Antibody binding was detected by enhanced chemiluminiscence (ECL Western Blotting Kit, Amersham) using horseradish peroxidase-conjugated immunoglobulin (Ig; Amersham) according to the manufacturer's instructions.

Flow cytometry

293T cells were removed from dishes 2 days after transfection by washing with calcium- and magnesium-free PBS. Cells were incubated at 4°C for 45 min with saturating amounts of fluorescein- or R-phycoerythrin (RE)-conjugated antibodies specific for CD4 or CD8 (Dako), washed twice with PBS, resuspended in 1% paraformaldehyde and analyzed by flow cytometry on a Becton Dickinson FACSCalibur. Cell-surface levels of the markers were expressed by multiplying the mean fluorescence of positive cells by the percentage of positive cells as previously described [66]. Analysis of gp41 on infected CEM-GFP cells was performed by incubating cells in a 1:10 dilution of T32 hybridoma supernatant, washing twice with RPMI plus 1% FCS, and then incubating cells with a 1:30 dilution of a PE-conjugated anti-mouse Ig (Dako), before fixation in 3% formaldehyde in PBS. Intact cells were selected by gating the population in the forward and side scatter.

Acknowledgements

We thank L. Barden and M. Loche for the artwork, and D. Littman, N. Landau, G. Nolan, and A. Gervaix for the gift of reagents. Special thanks to P. Earl for the gift of the T32 anti-gp41 hybridoma line. JL was the recipient of a Human Frontier Science Program Organization Fellowship. This work was supported by grants from the NIH (R37 AI 34306), from the Swiss National Science Foundation, and from the Gabriella Giorgi-Cavaglieri Foundation.

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