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Pseudotype HIV-1 Particles Carrying CD4

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=Abstract=

A defective HIV-1 helper virus DNA, pHyPC, was assembled by deleting the RNA packaging signal, *env*, *nef* and the 3'LTR sequences. HIV-1 like virus particles that carry the HIV-1 receptor, CD4 were generated by coexpression of pHyPC and plasmid DNAs encoding different chimeric CD4 proteins. The CD4 particles, sharing the CD4 ectodomain, precisely fused to different membrane anchors. CD4(+) particles specifically bound to HIV-1 Env expressing cells, but any signs of infection into these cells were not detected. Binding was only partially blocked by either polyclonal anti-CD4 antibodies or by high concentrations of soluble CD4. Surprisingly, CD4(+) particles also adsorbed to HeLa, CHO, NIH3T3 and COS-7 cells in the absence of HIV-1 Env expression. Adsorption was comparable in strength and speed to the highly specific CD4-Env interaction. CD4(−) particles exhibited only background levels of binding. Cell binding was CD4-dependent, but it was independent of the cell type from which the CD4(+) particles originated. Interestingly, CD4-dependent/Env-independent binding was only found when CD4 was present on virus particles. This suggests that the micro-environment of CD4 on virus particles uniquely expose this new cell binding activity. Its high affinity could explain in part why infection of Env (+) cells by CD4(+) particles was not detected. Further experiments will be required to evaluate whether this strong membrane interaction could represent one step in the multiple-step viral entry process.

Key Words: HIV-1, Packaging, CD4

INTRODUCTION

Targeted viral vectors could have broad application as analytic and therapeutic agents. Their development requires a detailed understanding of the mechanism of viral cytopathogenecity, viral assembly, receptor binding, membrane fusion, and entry [33]. Over the past years, avian leukosis [51], vesicular stomatitis virus (VSV) [45], human cytomegalovirus [31] and herpes simplex viruses [5,11] have been isolated that carry the HIV-1 receptor. The rationale of these experiments was target cells expressing the HIV-1 Env protein, and possibly to interfere with the replication of HIV-1 Env expressing

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cells. A targeted infection of HIV-1 Env expressing cells was reported, for the first time, with a Moloney murine leukemia virus (MuLV) after incorporation of a chimeric CD4 together with the ecotropic envelope protein into MuLV [30,43]. The rate of this targeted infection of HIV-1 infected HeLa cells, however, was very low and only detectable by PCR analysis.

The structural features of membrane proteins necessary for efficient insertion into viral envelopes are unknown. Depending on the virus, some sorting of proteins appears to occur at the site of virus budding [23,44].

Interactions between the cytoplasmic tail of the viral envelope protein and the viral matrix protein may play a role in some case but are not always essential [13,28]. Density and distribution of a particular viral protein within the plasma membrane also affect its insertion. Many studies on coinfections of different enveloped viruses have shown that viral glycoproteins are often exchanged very efficiently between viruses [6,44,49,52,53], which may simply be the result of a relatively high level of viral envelope protein expression as compared to cellular proteins. VSV grown in one cell type can be precipitated with antibodies raised against this cell type, but not by antibodies raised against another cell type [26,27]. Insertion of, for example, HLA Class I and Class II proteins [3,39] and cell adhesion receptors such as ICAM-1 glycoprotein into HIV-1 envelopes has been reported [4,14,15]. In fact, incorporation of cell adhesion proteins, such as ICAM-1 and LFA-1, can increase viral infectivity [14]. This demonstrates that cellular proteins can become structural and functional components of virus particles.

We previously inserted CD4 and a chimeric CD4/Gvsv molecules into VSV envelopes [45]. Surprisingly, both proteins were inserted into VSV particles with nearly equal efficiency. These observations were recently confirmed using recombinant VSV particles, which directly encode either CD4 or a chimeric CD4/G protein in their genomes [40]. Increased CD4 expression resulted in a high efficiency of a CD4 incorporation into VSV envelopes at a level similar to that of the VSV G protein itself. This demonstrates that much more free space exists on the surface of VSV envelopes than originally anticipated. These results also suggest that there is no general exclusion of foreign glycoproteins into VSV particles, although certain protein structures can negatively affect insertion, such as the cytoplasmic tail of the HIV-1 Env protein [42].

Our initial goal was to generate defective HIV-1 particles which carry the HIV-1 receptor in their envelopes in order to specifically target cells that express the HIV-1 Env protein. During HIV-1 infections, however, insertion of CD4 into HIV-1 particles is prevented by three known viral mechanisms. These mechanisms are very important for the production of infectious virus and viral spread, but they are detrimental to our goal and needed to be circumvented.

Downregulation of CD4 cell surface expression involves functions of the viral Vpu, Env, and Nef proteins [17,22,24,32,35,50]. CD4 downregulation through Vpu or Nef requires the cytoplasmic tail region of CD4 [7,17,34,38]. For receptor function such as Env binding and membrane fusion, the ectodomain of CD4 is sufficient [9,34]. For these reasons, we have assembled several chimeric CD4 molecules by replacing the transmembrane and cytoplasmic tail regions of CD4 with various membrane anchors.

In this communication, we describe the generation of the first HIV-1 particles, that carry the HIV-1 receptor in their envelopes, and we examine the targeting of these particles to HIV-1 Env expressing cells.

MATERIALS AND METHODS

Cells and cell culture

Monolayers of HeLa, HeLaT4 expressing hu-

man CD4 protein [29], HeLaS2 expressing CD4/gpi protein, COS-7, human 293 and NIH3T3 cells were grown at 37℃ in Dulbecco's minimal essential medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 1% each of penicillin and streptomycin (penicillin base 5000 units/ml-streptomycin base 5000 mg/ml). CHO-WT expressing HIV-1 envelope protein [37] and CHO-EE cells were grown in GMEM-S media. The cells were passed by treatment of confluent cell monolayers with 0.25% trypsin (GIBCO) for 5 to 10 min at 25℃, followed by centrifugation at 1000×g for 5 min to pellet the detached cells. Cell pellets were resuspended in growth medium and passed on to flasks. HeLaS2 cells, which constitutively express CD4/gpi, were established by retroviral transduction with the LA4SN vector [36] and selection in G418. The pool of G418 resistant cells was further enriched for CD4 expression by two rounds of positive selection using anti-human CD4 antibodies coupled to magnetic beads (Dynal A.S., Oslo, Norway). HeLaT4, CHO-WT and CHO-EE cells were obtained through the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Program.

Plasmids coding for chimeric CD4 proteins

(A) Plasmids expressing the CD4/gp41 fusion proteins were constructed by restriction enzyme digestion and PCR DNA fusion [45]. The fusion primers consisted of the carboxyl terminus of the CD4 ectodomain linked to the carboxyl terminal region of gp120, 20 amino acids upstream of the gp120/gp41 proteolytic cleavage site $^{L4\text{-}31250CD4}$-CCCCGGTGCAGCCA-ATGTTGAACCATTAGGAGTAGC-^{7750pN}. Template DNAs were pHD1 [44] and pNL4-3 [1]. The fused DNA was amplified by priming at the *Bst*EII site position 1250 of the CD4 gene with AAGCTTGGTTACCCAGGACC and by priming with GGAGGTGTATTAAGCTT-GTG at the *Hin*dIII site position 8145 within the gp41 gene.

The fused DNA was treated with *Hin*dIII and ligated with a *Hin*dIII fragment coding for the C-terminal region of gp41, positions 8145 to 8887 of pNL4-3. A 1680 bp fragment, containing part of CD4/gp41 coding region was removed by *Xba*I and *Bss*HII, blunted and cloned into pCR3 at the *Eco*RV site under control of the CMV promoter.

(B) CD4/G plasmid. The entire gene for CD4/G was excised from pCD4/G with *Xho*I and *Bss*HII. After filling in the ends with Klenow fragment, the blunted ended CD4/G was cloned into pCR3 at the *Eco*RV site to yield pCR3-CD4/G.

(C) CD4/Env plasmid. A 1085 bp fragment (*Bst*EII-*Xho*I) was removed from pHD1 and cloned into pCR3-CD4/G using the same sites to construct, pCR3-CD4/Env.

(D) CD4 plasmid. Part of the coding region of CD4 (603 *Bst*XI-1737 *Bam*HI) was removed from pT4B and cloned into the same sites in pCD4/G to yield pCR3-CD4.

Defective HIV-1 packaging construct

Construction of the packaging construct pHyPC required several deletions within the infectious HIV-1 clone pNL4-3 [1] and the replacement of the 3' LTR with a poly (A) site of SV40. Precise gene fusion was used to introduce several deletions in pNL4-3 by PCR and the following oligonucleotide primers: (I) A 654 bp fragment was amplified using primer #1(+) GAAGCGCGCACGGCAAGAGGCGAGGGG-CGGCGACTGGTGAGAGATGGGTGCGAG-AGCGTCGC, which introduced a 39 bp deletion between pos. 749-787 and primer #2(−) GGCCCTGCATGCACTGGATG. The fragment was cleaved with *Bss*HII and *Sph*I. pNL4-3 was cleaved with *Sph*I (pos.1404) and *Eco*RI (pos.5743) and a 4.3 kb fragment was isolated. These two fragments (647 bp and 4.3 kb) were ligated into pHD1 which had been cleaved with *Bss*HII and *Eco*RI. (II) Terminal primers #3: (+)CATAATAAGAATTCTGCAAC and #4: (−)CAAGTTAACAGCACTATTC were used

with fusion primers #5: (+)GGGATATTGATG-TCTGTAGAATAGGAGCTTTGTTCCTTGGG and #6: (−)CCCAAGGAACAAAGCTCCTAT-TCTACAGTCATCAATATCCC to generate a 1457 bp fragment, which contained a 1148 bp deletion in the Env region of pNL4-3 (pos. 6307-7755). This fragment was cleaved with *Eco*RI and HpaI. (III) A 240 bp fragment, containing the poly (A) site of SV40, was amplified using pJC119 [47] as a template and the following primers: Poly (A) primer 1:(+)TAG-CCCGGGATAAGATACATTGATGAGT; Poly (A) primer 2: (−)TAGGAATTCATCATAATC-AGCCATACCAC. This fragment was cleaved with *Sma*I and *Eco*RI. In the final step, the DNA clone from step (I) was cleaved with *Eco*RI and the fragments from step (II) and step (III) were cloned into (I) in a three piece ligation. The resulting clone was named pHyPC. It encodes all structural, regulatory and accessory proteins of HIV-1 except Env and Nef.

Immunofluorescent staining of CD4 proteins

Cells were grown for two days on glass coverslips prior to transfection with DNA encoding CD4 or chimeric CD4s using the calcium phosphate precipitation procedure. The cells were incubated for 24 hours, washed with cold PBS and fixed at room temperature for 20 min in 3% formaldehyde. Certain cells were permeabilized at room temperature by exposure to 0.4% Triton X-100 for 5 min. Nonspecific staining was blocked by incubating the cells for 60 min in the presence of 3% bovine serum albumin in PBS. Transfected cells were then incubated at room temperature for 40 min with mouse monoclonal antibody (Becton Dickinson Immunocytometry Systems, CA) or polyclonal sheep anti-human CD4 protein serum (the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Program). Each coverslip was then flooded with either donkey anti-mouse IgG or anti-sheep IgG, conjugated with fluorescent isothiocynate (FITC) and incubated for 40 min at room temperature. Washed coverslips were then mounted in 90% glycerol and 10% PBS containing 1% 3,4,5-trihydroxybenzoic acid N-propylester to reduce photobleaching. Cellular distribution of immino-fluorescence was determined with a transmission microscope and epifluorescent ultraviolet light source.

Generation of defective HIV-1 particles

HeLa, HeLaT4, HeLaS2, COS-7, 293 and NIH3T3 were grown to 80% confluence for 24 hours at 37℃ on 35 mm dishes in DMEM containing 10% FBS. The cells were cotransfected with 2.5 μg of pHyPC DNA and 2.5 μg of CD4 DNA using the calcium phosphate precipitation method (Promega). Two days after cotransfection, the medium was collected, clarified by passing through a 0.45 μm filter and p24 antigen in the supernatant was quantitated using a HIV-1 p24 ELISA kit (Cellular Products Inc.).

Immunoprecipitation of defective HIV-1 particles

Up to 100,000 pg p24/ml of defective viral particles were pre-incubated at room temperature for 30 min with 50 μl of Protein-A-Sepharose, 100 mg/ml in 10 mM Tris-HCl, pH 7.4 (Sigma Chemical Co., St. Louis). CD4 containing particles were precipitated by incubating with 0.5 mg/ml of polyclonal antibody to CD4 antigen (the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Program), at room temperature for 2 hours or overnight at 4℃. Complexes were bound to Protein-A-Sepharose and pelleted by centrifugation. The particles were disrupted using the lysis buffer from the p24 ELISA kit (Cellular Products Inc.) and released p24 proteins were quantitated by ELISA.

Enzyme-linked immunosorbent assay for p24 and CD4 proteins

The concentration of HIV-1 p24 protein was

determined using a p24 ELISA kit following the supplier's procedures (Cellular Products Inc.). The amount of CD4 was analyzed by a modified ELISA: 100 μl of the fraction sample were added into wells of a 96 well Immuno-4 plate (Dynatech Laboratories, Inc.) next to soluble CD4 protein standards (Intracel Corporation). The plates were incubated overnight at 4℃ in a moist chamber, washed with 0.05% of Tween 20, 10 mM Tris, 0.01% Thimerosal and blocked at room temperature for two hours with 3% BSA. After antigen adsorption, 50 μl of anti-CD4 polyclonal antibodies (5 μg/ml in PBS) were added and incubated for an additional hour at room temperature in the presence of 0.15 μg of alkaline phosphatase-conjugated anti-sheep IgG (Jackson Immuno Research Laboratories, Inc.). After the incubation, 50 μl of nitrophenylphosphate (1 mg/ml in 10 mM diethanolamine, pH 9.5, and 0.5 mM $MgCl_2$) was used as a substrate. The reaction was stopped by addition of 0.1 M EDTA and colorometrically read at 405 nm.

Centrifugation and fractionation of the defective viral particles

Ten milliliter of the medium from transfected cell was collected and passed through a 0.45 μm filter. Defective particles were concentrated by centrifugation onto 65% sucrose pad at 150,000×g for 60 min. The concentrated particles were resuspended in 0.5 ml of PBS and overlaid onto 4.5 ml of a continuous gradient of 15~60% sucrose in PBS (pH 7.5). The gradients were centrifuged at 120,000×g for 2 hours and then fractionated into 0.35 ml aliquots. The concentration of p24 and CD4 proteins were analyzed in parallel by ELISA.

Virus adsorption assay

Defective viral particles were generated as described above. The recipient cells were grown to 85% confluence. HeLa, COS-7 and NIH3T3 cells were infected at a multiplicity of infection of 5 with a vaccinia virus (vPE16) expressing the HIV-1 Env (10) protein and vTF7, expressing T7 polymerase [16] as a control. Infected cells were incubated at 37℃ for 24 hours before harvest. CHO-WT cells expressing the HIV-1 envelope protein and CHO-EE cells control cells were grown to 85% confluence. The cells were detached using a cell scraper and passed through a #16 needle, washed three times with PBS and resuspended in DMEM containing 10% FBS. These cells were used for virus adsorption. In some adsorption experiments, the cells were fixed at room temperature for 20 min with 3% formaldehyde.

Virus attachment was studied using a modified procedure described by Roelvink et al. 1996 [40]. 10^5 cells were incubated with particles containing 10,000 to 20,000 pg/ml of p24. In the competition assay, the particles or recipient cells were pre-incubated with polyclonal antibody to CD4 protein or with polyclonal antibody to gp120 antigen (the National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Program). After incubation, the cells were pelleted, and the pellet was washed three time with medium containing 10% FBS. Cell pellets were resuspended in 200 μl DMEM. Adsorbed particles were solubilized by lysis buffer and released p24 was quantified by ELISA.

RESULTS

Structure and expression of CD4 and chimeric CD4 proteins

Using PCR gene fusion techniques, several chimeric CD4 proteins have been assembled as schematized in Figure 1. Each protein contains 397 amino acids of the ectodomain of the CD4 molecule including the signal peptide. The CD4 ectodomain was precisely fused to either a gpi anchor, the transmembrane and cytoplasmic tail region of the VSV G protein [45] or the HIV-1 Env protein [34,44]. The proteolytic cleavage site was either left unchanged or it was modified by site specific mutation.

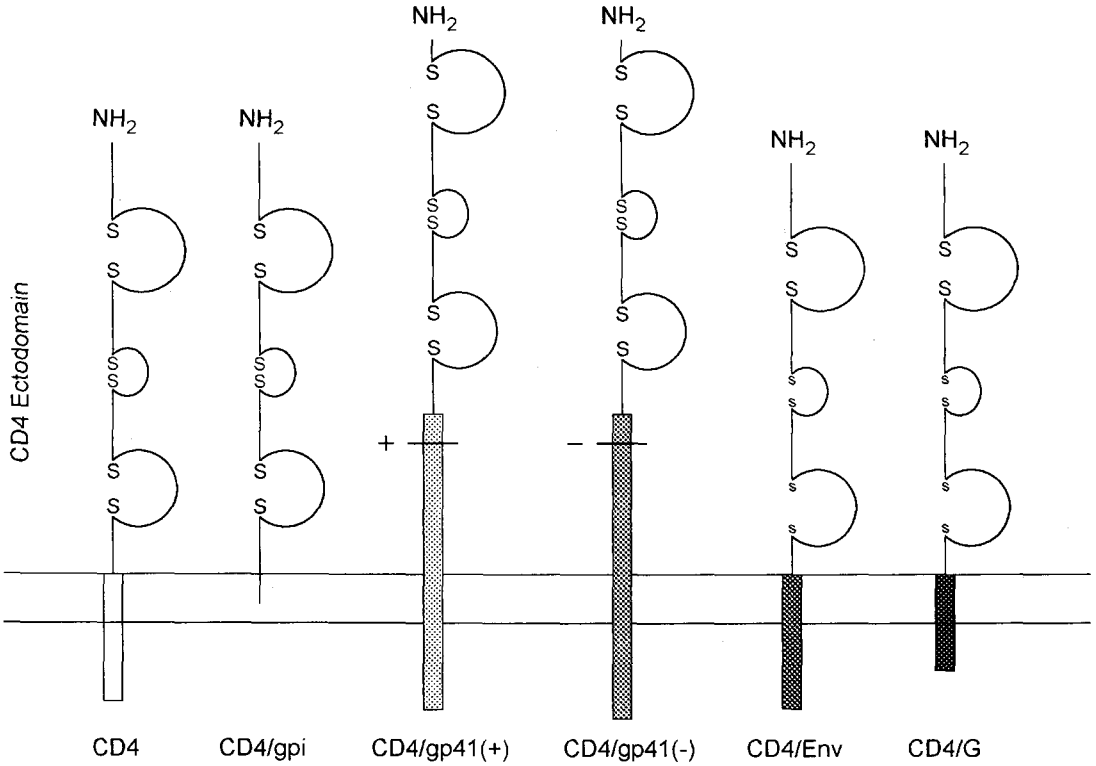


Figure 1. Chimeric CD4 constructs. The CD4 ectodomain region was precisely fused to different membrane anchors that consisted either of the glycosyl-phosphatidylinositol anchor (CD4/gpi), a HIV-1 gp41 protein with a NH_2 terminal extension of 20 amino acids into the gp120 region with (+) or without (−) the putative proteolytic cleavage site (CD4/gp41) or the transmembrane and cytoplasmic regions of either HIV-1 Env (CD4/Env) or VSV G protein (CD4/G), respectively.

The wild type and the chimeric CD4 protein were cloned into the pCR3 eukaryotic gene expression plasmid under control of the CMV early promoter. Following transfection of the DNA's, the six proteins were analyzed by Western blot using antibodies directed against the ectodomain of CD4. Only one major protein from each cell extract reacted with anti-CD4 antibody. Degradation of chimeric CD4 proteins was not observed. All six CD4 related proteins migrated on the gel according to their expected molecular weight. Expression levels differed among the individual protein constructs. The CD4/gp41 proteins were usually expressed at lower levels.

A similar difference of expression levels was observed by immunofluorescent staining of cells after DNA transfection. Cells with or without permeabilization showed intracelluar and cell surface stain, respectively. The staining intensities were slightly different between the chimeric proteins. All proteins appeared to be transported efficiently to the plasma membrane and are all potentially available for incorporation into virus particles.

The chimeric CD4 proteins are functional receptors

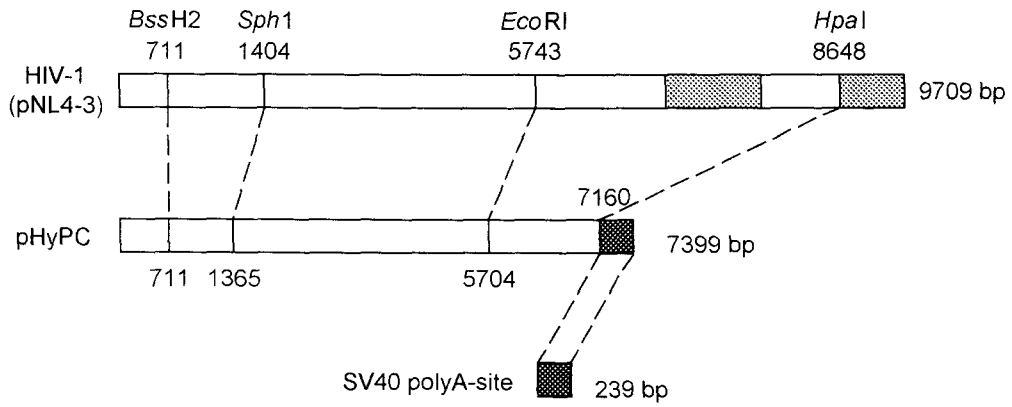
Functionality of the chimeric CD4 protein was tested using a syncytia forming assay. Coexpression of the chimeric CD4 protein with HIV-1 Env protein in HeLa cells using vaccinia virus derived T7 RNA polymerase, resulted in syncytia formation, except for both of the

CD4/gp41 proteins (data not shown). Expression levels of the CD4/gp41 proteins were usually lower. We did not observe fusions with these proteins yet. It has previously been reported that syncytia formation requires the ectodomain of CD4 while the normal membrane anchor and cytoplasmic tail can be replaced [9,44, 45]. This is consistent with our findings here with CD4/G, CD4/gpi and CD4/Env.

Incorporation of CD4 into defective HIV-1 particles

For the generation of defective HIV-1 viruses that carry CD4, we have constructed a defective HIV-1 packaging construct, pHyPC (Figure 2). The infectious DNA clone of HIV-1, pLN4-3, was altered by deleting part of the HIV-1 packaging signal, most of the Env and all of the Nef regions, and by replacing the 3'LTR with a SV40 polyadenylation signal. All other sequences, including the genes for Tat and Rev and the RRE, were left intact. This DNA, together with a mini HIV-1 genome encoding a neomycin resistance gene, was used to stably transduce cells for neomycin resistance after pseudotying the particles with the vesicular stomatitis virus glycoprotein (data not shown). This demonstrated that the helper virus construct, pHyPC, was fully functional in expressing Env(−) HIV-1 particles which can be used for pseudotype formation, membrane studies as well as gene transfer.

Cotransfections of pHyPC with the chimeric CD4 DNAs into HeLa cells yielded approximately 15~25 ng of p24/ml in two days, as a measure of virus particle release. At two days post transfection approximately half of the p24 was found in the cell supernatant and the other half was cell associated (Table 1). Using a polyclonal anti-CD4 antibody, the HIV-1 like particles in the cell supernatants were immunoprecipitated as shown in Table 1. Between 11% and 45% of the supernatant p24 were precipitated with anti-CD4 antibody. There was a rough correlation between the amount of CD4 protein expressed in transfected cells and the



Deletions in pNL4-3: 1. pos. 749-787 Part of packaging signal
2. pos. 6307-7755 Part of Env
3. pos. 8651-9709 Part of Env, Nef, 3' LTR

Figure 2. pHyPC helper virus construct. Three deletions were made in the infectious clone of HIV-1, pNL4-3 as indicated. pHyPC provides all the structural proteins of HIV-1 except Env and Nef and it is able to form viruslike particles.

Table 1. Generation and immunoprecipitation of CD4(+) HIV-1 particles

Virus particles[a]	p24 antigen (ng)[b]			
	Intracellular (I)	Supernatant (S)	S/(I+S)[c]	anti-CD4 (P)
pHyPC	31.1	24.9	44	4.5
pHyPC-CD4	24.0	20.5	46	9.4
pHyPC-CD4/gpi	19.1	17.1	47	12.2
pHyPC-CD4/Env	22.5	16.8	43	10.8
pHyPC-CD4/G	28.2	26.0	48	16.1
pHyPC-CD4/gp41(+)	24.0	18.9	44	6.6
pHyPC-CD4/gp41(−)	22.0	21.0	49	7.8

[a] Human 293 cells were cotransfected with pHyPC DNA and the various chimeric CD4 DNAs.
[b] The amounts of intracellular (I), extracellular (S) and anti-CD4 precipitable (P) p24 antigen were determined by ELISA.
[c] The amount of extracellular viral p24 as a percentage of the total amount of p24 produced

number of virus particles that could be precipitated. The precipitation of particles carrying the wild type CD4 protein in this particular experiment was somewhat lower than anticipated from the staining pattern. Particles carrying the chimeric CD4/gp41 proteins also showed lower precipitation, consistent with lower CD4/gp41 expression levels. These results suggested that incorporation of the chimeric CD4 proteins into HIV-like particles seems to correspond more to their expression levels instead of the type of membrane anchor.

As compared to DNA cotransfections, we obtained even higher yields (over 100 ng/ml) of CD4 bearing virus particles by single transfection of pHyPC into either HeLaT4 or HeLaS2 cell lines, which constitutively express high levels of either CD4 or CD4/gpi on their surface. Sucrose gradient analysis of supernatants from pHyPC transfected cells showed a peak of p24 antigen in fraction No. 5, which was anticipated to contain HIV-1 or the defective HIV-1 like particles (Figure 3A). The position of the p24 peak was the same, for particles derived from either HeLa, HeLaT4 or HeLaS2 cells.

It should be noted that less than 10% of the total amount of p24 was found spread among fractions towards the top of the gradient. Approximately 90% of the total p24 antigen was generally found in the peak fractions, presumably all incorporated into virus particles. This was confirmed by pretreating the particles with non-ionic detergent (0.4% Triton) prior to centrifugation. Detergent treatment totally solubilized the envelope of the virus, causing all of the p24 antigen to be shifted to the top of the gradient (Figure 3A).

To verify that these particles contained CD4 or CD/gpi, the same gradient fractions were also analyzed by ELISA for the presence of CD4 antigen (Figure 3B). The peak for CD4 antigen was also found in gradient fraction No. 5. A calculation of the CD4 to p24 ratio from the same gradient fractions reveals that each HIV-1 particle contained approximately 100 molecules of CD4 or CD4/gpi.

Adsorption of CD4(+) particles to HIV-1 Env(+) and Env(−) expressing cells

HIV-1 like particles were harvested after cotransfections of pHyPC with the different CD4 DNA constructs. Equal amounts of p24 antigen were used for cell adsorption. Prior to virus adsorption, the cells were infected with a recombinant vaccinia virus expressing either the HIV-1 Env protein or T7 RNA polymerase as a control for vaccinia virus. The vaccinia virus infections gave rise to HeLa cells with or without a functional HIV-1 Env on the cell

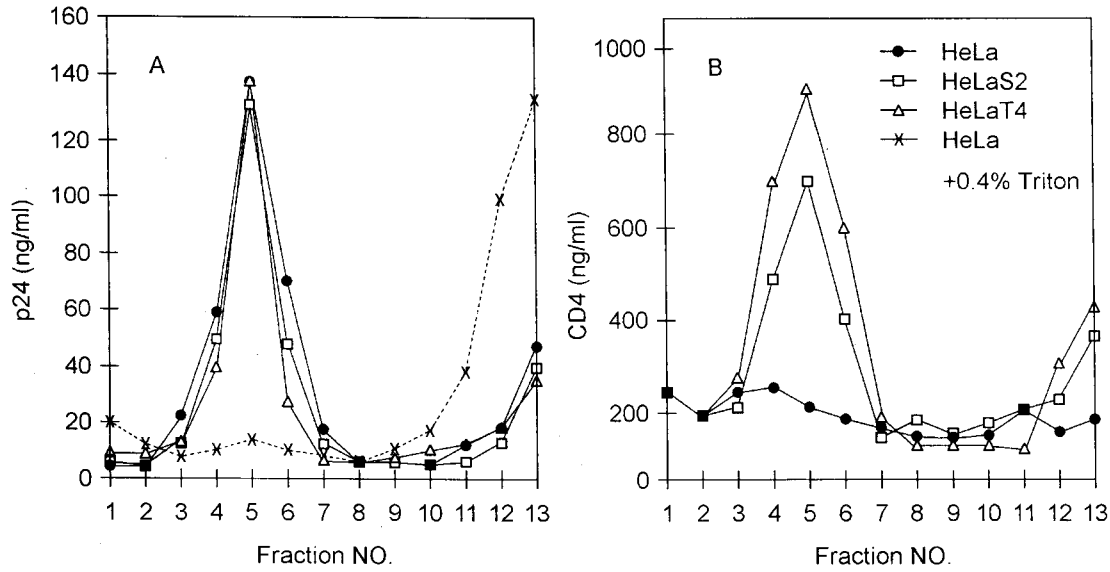


Figure 3. Separation of CD4(+) HIV-1 particles on sucrose gradients. HIV-1 like particles with or without CD4 or CD4/gpi were generated by cotransfecting HeLa, HeLaT4 or HeLaS2 cells, respectively with H. The virus was concentrated by centrifugation onto a 65% sucrose pad and then fractionated on a 15~60% sucrose gradient. A portion of the virus particles generated in HeLa cells were pretreated with Trion X-100 to solubilize the viral membrane. The amounts of p24 antigen (panel A) and CD4 (panel B) in each gradient fraction were determined by ELISA.

surface as determined by immunofluorescent staining (data not shown). In parallel, Chinese hamster ovary cells with (CHO-WT) or without (CHO-EE) constitutive expression of the wild type HIV-1 Env protein were used as recipient cells for CD4(+) particles.

As shown in Figure 4, pHyPC particles without CD4, adsorbed at a very low affinity to both CHO and HeLa cells, independent of the presence of HIV-1 Env protein. It required an incubation period of about 30 min to reach appreciable levels of binding. As expected, CD4 (+) particles that carried one of the five different CD4 proteins (Figure 1) strongly bound to Env(+) HeLa and CHO cells. In contrast, to CD4(−) particles, however, CD4(+) surprisingly exhibited considerable binding to both HeLa and CHO cells as well, even in the absence of Env. Within one minute at 4℃, levels of binding were obtained that were reached by CD4(−) particles only after 30 min. The kinetics of adsorption were almost identical for all CD4(+) particles tested. Adsorption to Env(+) recipient cells was always higher, however, roughly half of this cell binding may have been contributed by this unexpected CD4-dependent/ Env-independent adsorption.

Since the binding of CD4 to Env can be greatly enhanced by raising the temperature above 13℃ [9], we also carried out virus adsorption at room temperature and at 37℃. We did not observe a selective increase in the efficiency of the CD4-mediated, Env-dependent adsorption as compared to the Env-independent adsorption of CD4(+) particles to cells. Both were equally enhanced by increasing temperature (data not shown).

Anti-CD4 antibodies and CD4 did not totally block adsorption of CD4(+) particles to Env(−) cells

This surprisingly high affinity of CD4(+) particles to Env(−) cells indicated that the ectodomain of CD4 may function as another li-

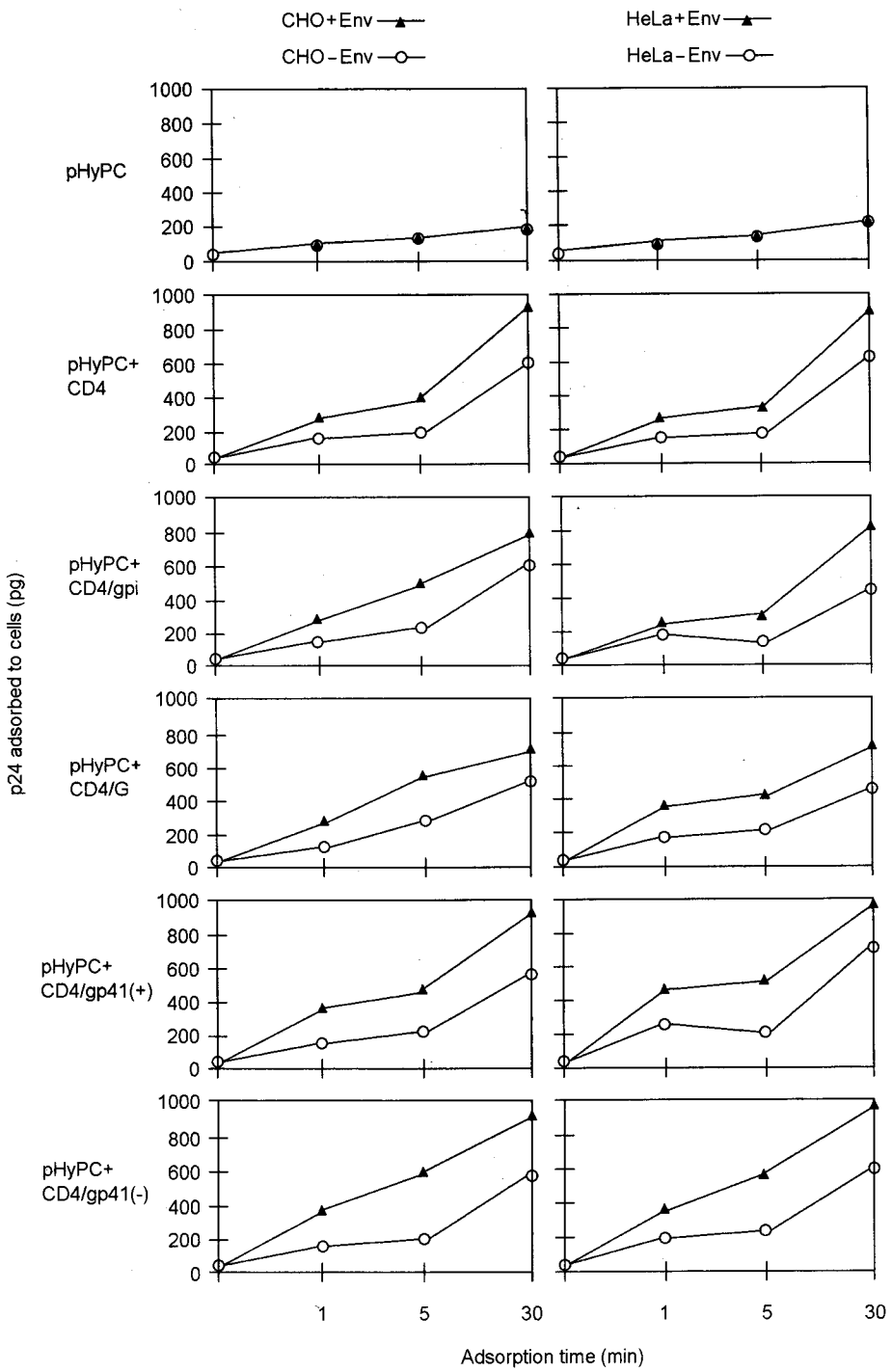


Figure 4. Adsorption of CD4(+) particles to CHO and HeLa cells. CD4(+) particles were generated by cotransfection of pHyPC and either pCR3-CD4, pCR3-CD4/gpi, pCR3-CD4/G, pCR3-CD4/gp41(+) or pCR3-CD4/gp41(−), respectively. Equal numbers of particles (10000 pg of p24) were added to approximately equal numbers of suspended recipient cells ($1x10^5$). One fifth of the mixture was used for measuring p24 by ELISA. The recipient cells were: CHO cells (-○-) or CHO cells that constitutively express the HIV-1 Env protein (-▲-), and HeLa cells which were infected with either a vaccinia virus recombinant expressing HIV-1 Env protein (-▲-) or T7 RNA polymerase as a negative control (-○-). Virus adsorption to cells was carried out at 4℃ for the indicated times.

gand to the recipient cells or, alternatively, that an as yet unidentified protein and/or lipid moiety may have been recruited by CD4. This moiety may specifically bind at high efficiency to both recipient cell types tested (CHO and HeLa). This unique property of CD4(+) particles was never observed with pHyPC particles that lacked CD4.

To distinguish between these possibilities, CD4(+) particles were preincubated with an excess of polyclonal antibody to CD4 prior to cell adsorption. Figure 5A shows that binding attributable to the CD4: Env interactions from both CD4(+) and CD4/gpi(+) particles, could be completely blocked with anti-CD4 antibody. However, there was residual binding, that was dependent on the presence of CD4 in the particles but was not mediated by Env. Particles generated in HeLa cells only showed a constant, much lower level of adsorption at all antibody concentrations. At the same concentrations, anti-CD4 antibody had no affect on the Env-independent binding of CD4(+) and CD4/gpi(+) particles to HeLa cells (Figure 5B). CD4

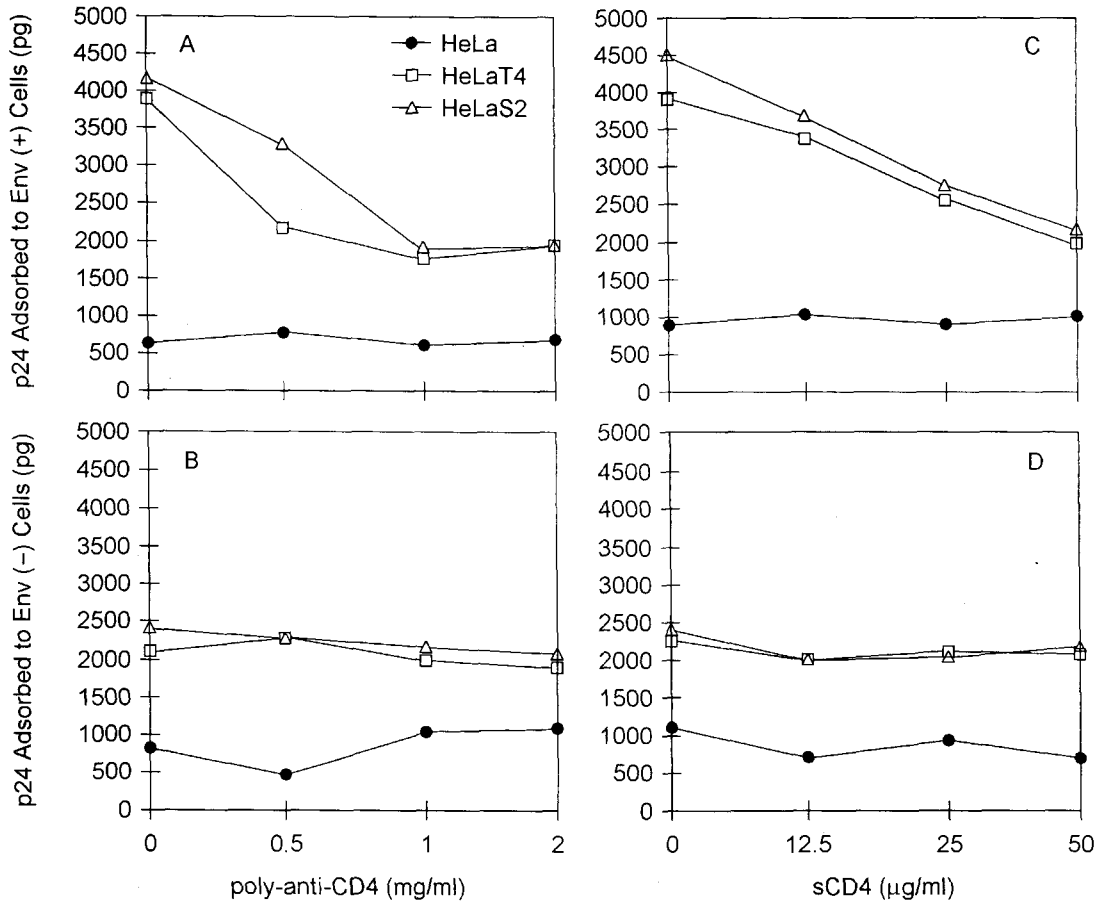


Figure 5. Partial inhibition of CD4(+) virus adsorption by anti-CD4 antibody and soluble CD4. Equal amounts of CD4(+) particles generated in HeLaT4 and HeLaS2 cells as well as CD4(−) particles generated in HeLa cells were adsorbed to HeLa cells expressing HIV-1 Env protein (Env+) (panels **A** and **C**) or T7 RNA polymerase as a control (Env−) (panels **B** and **D**) from recombinant vaccinia viruses. Virus adsorption was carried out at 4℃ for 30 min after pretreatment of the virus or the cells with either poly-anti-CD4 antibody or soluble CD4 for 16 hours at 4℃ at the concentrations indicated.

Table 2. Generation and adsorption of CD4(+) HIV-1 particles to various cell types

Producer cell		Recipient cell-adsorbed p24 (%)[c]							
Cell[a]	pHyPC particles[b]	HeLa	HeLa+Env	COS	COS+Env	3T3	3T3+Env	CHO	CHO+Env
HeLa	CD4+	8	10	9	11	10	11	10	9
HeLa	CD4/gpI+	42	94	35	102	38	92	42	96
HeLa		46	100	42	100	39	100	40	100
COS	CD4+	11	10	10	16	8	14	8	12
COS	CD4/gpI+	33	79	76	165	77	86	62	124
COS		35	100	75	100	70	100	50	100
3T3	CD4+	10	11	13	10	8	15	12	13
3T3	CD4/gpI+	38	101	45	150	49	131	46	133
3T3		38	100	53	100	69	100	50	100
HeLaT4	CD4+	38	100	36	100	50	100	64	100
HeLaS2	CD4/gpI+	40	100	46	100	56	100	58	100

[a] These cells were transfected or cotransfected

[b] Transfections always included pHyPC DNA together with pCR3-CD4 or pCR3-CD4/gpi DNA as indicated, except for HeLaT4 and HeLaS2 cells that constitutively express CD4 and CD4/gpi, respectively.

[c] Equal amounts of viral p24 were added for 30 min at 4℃ to the recipient cells. Recipient cells were infected with a vaccinal virus recombinant expressing either the HIV-1 Env protein (+Env) or the T7 RNA polymerase as a control. CHO cells that constitutively express HIV-1 Env or not were also used as recipient cells. The amounts of p24 adsorbed to the cells were normalized with the amount of CD4/gpi particles adsorbed to Env(+) cells in each block arbitrarily set at 100%

(+) and CD4/gpi(+) particles showed a high affinity to HeLa cells and unexpectedly this affinity is similar to the CD4 to Env interaction itself.

Competitive inhibition of CD4 to Env binding in the presence of soluble CD4 is shown in Figure 5C. Even at the highest concentration of sCD4 used, there were similar levels of residual particle adsorption as was found in the presence of high anti-CD4 antibody concentrations. CD4 at very high concentration did not totally block the binding of CD4 particles to Env(+) cells (Figure 5C). Again, there was no effect of sCD4 on the binding CD4 particles to Env(−) HeLa cells (Figure 5D).

A similar inhibition of CD4 to Env binding was observed after the addition of a small peptide that corresponds to the Env binding site on CD4 [25].

Adsorption of CD4(+) particles generated in different producer cells to different recipient cells

The number of particles were adjusted to 20,000 pg/ml of p24, were adsorbed to equivalent numbers of HeLa, COS-7, NIH3T3 and CHO cells, respectively. HeLa, COS-7 and NIH-3T3 cells expressing the HIV-1 Env were prepared by prior infection with a vaccinia virus recombinant encoding HIV-1 Env. CHO-WT cells constitutively express HIV-1 Env. Control cells without Env expression were either CHO-EE, HeLa, COS-7 or NIH3T3 cells which were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase.

CD4(+) particles were generally adsorbed to cells for 30 min at 4℃. As shown in Table 2, all virus adsorption to cells was greatly dependent on the presence of the ectodomain of CD4. Env-independent viral adsorption occurred with CD4(+) particles that were generated from any

of the producer cells, and CD4(+) particles adsorbed well to all of the recipient cell types tested. Virus binding was increased in most cases when the recipient cell also expressed HIV-1 Env. In some cases, binding activities appeared relatively higher, when the particles were generated from the same type of cell as the recipient cell. For example, binding activities of CD4/gpi/HIV-1 particles from COS-7 and NIH-3T3 cells were 75% and 70% to COS-7 and NIH3T3 cells. The same particles, however, had 35% and 38% binding activities to HeLa cells.

Only CD4 on virus particles facilitates Env-independent adsorption

To see whether CD4(+) cells would bind HIV-1 particles that lack the Env protein, we generated pHyPC in HeLa cells and tried to adsorb these particles to either HeLaT4 and HeLaS2 cells. We did not detect any significant binding of CD4(−) particles to either one of these cells, while CD4(+) particles adsorbed to both CD4(+) as well as CD4(−) cells (data not shown). This demonstrates that the CD4-dependent/Env-independent binding requires the CD4 ectodomain to be located on the viral membrane.

DISCUSSION

HIV-1 Env protein is not required for HIV-1 particle formation [18,46]. HIV-1 readily forms pseudotypes with other virus envelope protein, for example, VSV G protein. HIV-1 carrying VSV G protein can reach levels of viral infectivity that are 100 fold greater than that of wild type virus itself [37]. In the study presented here, we have inserted a modified cellular protein, the HIV-1 receptor, into the envelope of HIV-1 particles. To increase the efficiency of CD4 protein insertion, we have circumvented the HIV-1 induced downregulation of CD4 expression on the plasma membrane by deleting several regions in our HIV-1 helper virus construct, pHyPC, including Env and Nef. Several chimeric CD4 glycoproteins were generated by replacing the normal transmembrane and cytoplasmic tail regions of CD4 with those of the VSV G and the HIV-1 Env, as well as with a gpi anchor (Figure 1).

It is difficult to provide precise numbers about the insertion efficiency of each CD4 construct into HIV-1 particles. From the amount of CD4(+) virus immunoprecipitations and the amount of CD4 cell surface expression, we estimates that membrane insertion of a particular CD4 molecule was primarily dependent on the level of cell surface expression. Most of the cell adsorption studies were carried out with CD4(+) virus generated in HeLaT4 and HeLaS2 cells which show relatively high levels CD4 and CD4/gpi expression on the cell surface. While the proteolytic cleavage site in CD4/gp41 was not studied in detail in this report, a free ectodomain of CD4 which could have resulted from cleavage was not observed in Western blots.

We have made several attempts to specifically infect Env(+) cells using CD4(+) particles. Infections were followed by transfer of a hygromycin resistance marker gene to cells that were persistently infected with HIV-1 or HIV-2 viruses, or to CHO-WT cells that express the HIV-1 Env protein. While resistance was transferred by particles pseudotyped with the VSV G protein, and CD4(+) particles were not able to stably transduce the resistance gene (data not shown). The sensitivity of this assay was approximately 1/100 of the virus pseudotyped with VSV G protein.

We conclude that a topographic reversal of the location of the viral envelope protein and viral receptor/co-receptor from cellular to viral membranes does not lead to a functional interaction between receptors and ligands that would result in membrane fusion infection. Although specific targeting through CD4 was accomplished, there may be several reasons why membrane fusion and infection did not occur.

Cells expressing CD4 and HIV-1 Env are

able to form large syncytia involving large membrane segments. The concentration or the local density of CD4 in virus particles may differ and it may not be sufficient to allow fusion of the viral and cellular membranes. Another possibility why infection did not occur, may in part involve the newly discovered CD4-dependent Env independent cell binding activity of CD4(+) particles, which could have blocked viral infection.

The presence of the CD4 ectodomain was essential for cell binding, but binding was not inhibited by two different polyclonal anti-CD4 antibodies at concentrations that blocked regular CD4-Env interactions (Figure 5). These new adsorption activities were not observed with particles containing viral glycoproteins, for example, VSV G or HIV-Env (data not shown).

These results suggest that the CD4 ectodomain may not be fully responsible for this cell binding, however, we can not completely rule out that the two different polyclonal antibodies that were used may not have covered all regions of CD4 equally well. Attempts to block the potential cellular binding sites by preincubating the cells with large amounts of either soluble CD4, or a synthetic CD4 peptide, did not totally abolish virus binding to either Env (+) or Env(−) cells, even at concentrations when the CD4-Env interaction itself was drastically blocked by both. This again indicates that while Env independent ectodomain may not be physically involved in cell binding. It was not critical for cell binding whether the CD4(+) virus was generated in human, African green monkey, Chinese hamster or mouse cells. Cell adsorption of CD4(+) virus also occurred when these cell types were recipient cells (Table 2). In contrast, CD4(+) cells do not interact with spikeless particles, indicating that CD4 must be present on the virus particle for this binding to occur.

These observations suggest that if cell adsorption does not involve the CD4 ectodomain directly, an ubiquitous membrane component found on many cell types and across species, such as glycolipids or phospholipids, may interact with another ubiquitous component on the recipient cells. In the virus particle, this membrane component with cell binding activity would somehow have to be recruited by ectodomain of CD4. It is not clear how the chimeric CD4 proteins with such vastly different structures could facilitate this.

Alternate receptors for HIV-1 particles have been described such as mannose binding-lectins [41]. Also, galactosyl ceramide has been identified as an alternate HIV-1 receptor on neuronal cells [8,19,20,21]. These alternate routes of viral entry all require the HIV-1 Env, which differ from the binding activity described here that is independent of Env.

CD4(+) cells obviously must contain this binding activity because they are the source of the budding virus, but the cells do not express this unique binding activity themselves. Therefore, it appears that the active binding site may not be exposed on the cell surface. In the microenvironment of the viral membrane, however, it may be unmarked. Alternatively, at the site of viral budding, a sorting of membrane proteins may occur which enriches for certain modified CD4 proteins or CD4 associated proteins or lipids that participates in the new activity.

In summary, our study on the insertion of CD4 into HIV-1 particles revealed surprising new affinities of the virus which have important implications for the mechanism of viral entry as well as for the future design of targeted enveloped viruses.

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