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Defective Interfering HIV-1 Pseudotypes Carrying Chimeric CD4 Protein

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Chimeric CD4 proteins were assembled. They contained the entire CD4 ectodomain that is linked to different membrane anchors. Membrane anchors consisted of either glucosyl phosphatidyl inositol (gpi), the transmembrane and cytoplasmic regions of HIV-1 Env protein, or the vesicular stomatitis virus G glycoprotein, respectively. The HIV-1 co-receptor CXCR4 and CD4 were independently inserted into viral envelopes. We compared the insertion of six different CD4/CXCR4 constructs into HIV-1 envelopes, as well as their functionality in targeting and specific infection of cells that constitutively express the HIV-1 Env protein. All of the six different HIV-1 (CD4/CXCR4) pseudotypes were able to transduce Env (+) cells at similar efficiency. In addition, stable transduction of the Env (+) recipient cells demonstrated that all chimeric proteins were functional as receptors for Env when inserted into HIV-1 envelopes. In fact, these results demonstrate for the first time a stable transduction by a targeted HIV-1 pseudotype virus.

Keywords: HIV-1 pseudotype, CD4, CXCR4

Introduction

We previously inserted receptor (CD4) and co-receptor (CXCR4) molecules into VSV envelopes. Surprisingly, both proteins were inserted into VSV particles with nearly equal efficiency. These observations were recently confirmed using recombinant VSV particles, which directly encodes either CD4 or a chimeric CD4 protein in their genomes. The increased CD4 and CXCR4 expression resulted in a high efficiency of CD4 incorporation into VSV envelopes at a level that was similar to that of the VSV G protein itself. This demonstrates that much more free space exists on the surface of VSV envelopes than was originally anticipated. These resulted 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. These pseudotypes were able to specifically infect cells that express HIV-1 or VSV envelope protein. Depending on the inserted co-receptor, even the selective infection of cells that express the T cell tropic and macrophage tropic Env HIV-1 strain was maintained (Endress *et al.*, 1997).

The mechanism for the insertion of foreign viral or cellular glycoproteins into viral envelopes is poorly understood. There may be different requirements for different viruses, as has been shown between the closely related VSV and rabies virus. With the rabies virus, the origin of the membrane anchor for CD4 and CXCR4 was critical for insertion (Mebatsion *et al.*, 1997). With VSV, the type of membrane anchor was not critical for CD4 insertion (Schubert *et al.*, 1992), but it was critical for the insertion of HIV-1 Env into VSV particles (Owens *et al.*, 1993; Reiser, 2000). How these proteins are sorted at the site of virus budding is still unclear. Insertion of foreign viral and cellular membrane proteins into envelopes have been described for VSV (Little *et al.*, 1983; Schnell *et al.*, 1996) and HIV-1 (Schnell *et al.*, 1996; Schubert *et al.*, 1992), which appears to be very tolerant and can even insert cell adhesion molecules (Hioe, *et al.*, 1998; *Orentas et al.*, 1993).

In this communication, we focus on the generation of functional HIV-1 (CD4/CXR4) pseudotypes, and ask whether there are structural restrictions for the functional insertion of chimeric CD4 proteins with different membrane anchors into HIV-1 envelopes. Is the insertion of CD4 or CXCR4 cooperative, as was described for the rabies virus (Mebstsion *et al.*, 1997)? Is the insertion of CXCR4 from basal cellular levels into HIV-1 pseudotypes sufficient to allow the generation of targeted pseudotype particles? Do these pseudotypes specially target and transduce Env (+) cells, and for the first time confer neomycin resistance?

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Materials and Methods

Cells and cell culture Monolayers of HeLa, HeLaT4 expressing the human CD4 protein, HeLaS2 expressing the CD4/gpi protein, COS-7, human 293, and NIH3T3 cells were grown at 37°C in Dulbecco's minimal essential medium (DMEM; GIBCO) that was supplemented with 10% fetal bovine serum (FBS), 1% each of penicillin and streptomycin (penicillin base 5000 units/ml-streptomycin base 5000 mg/ml). TF228, expressing the HIV-1 envelope protein, and BJAB 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°C, followed by centrifugation at 1000 g for 5 min in order to pellet the detached cells. The cell pellets were resuspended in a growth medium and passed on to flasks. The selection of stable NIH3T3 and TF228 transfectants was carried in the presence of 400 μg neomycin/ml.

Plasmids coding for chimeric CD4 proteins (A) Plasmids that expressed the CD4/gp41 fusion proteins were constructed by restriction enzyme digestion and PCR DNA fusion. The fusion primers consisted of the carboxyl terminus of the CD4 ectodomain that was linked to the carboxyl terminal region of gp120, 20 amino acids upstream of the gp120/gp41 proteolytic cleavage site 1250CD4-CCCCGGTGCAGCCAATGTTG/AACCATTAGGAGT AGC-7750pNL4-3. Template DNAs were pHD1 (Schubert *et al.*, 1995) and pNL4-3 (Adachi *et al.*, 1986). The fused DNA was amplified by priming at the *Bst*E II site position 1250 of the CD4 gene with AAGCTTGGTTACCCAGGACC and by priming with GGAGGTGTATTAAGCTTGTG at the *Hind* III site position 8145 within the gp41 gene.

The fused DNA was treated with *Hind* III and ligated with a *Hind* III fragment that was coded for the C-terminal region of gp41, positions 8145 to 8887 of pNL4-3. A 1680bp fragment, containing part of the CD4/gp41 coding region, was removed by *Xba* I and *Bss*H II, blunted and cloned into pCR3 at the *Eco*R V site under control of the CMV promoter. CD4/gp41-2 was constructed in the same way as CD4/gp41, except the coding sequences for Ile and Glu (ATT and GAA) were deleted in the fusion primers. CD4/gp41 misses two amino acids, which belong to the proteolytic cleavage site.

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

(C) CD4/Env plasmid. A 1085bp fragment (*Bst*E II-*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*X I-1737 *Bam*H I) was removed from pT4B and cloned into the same sites in pCD4/G to yield pCR3-CD4.

(E) pCR3-CD4/gpi was constructed by excising the GPI anchored CD4 chimeric gene out of the retroviral vector LA4SN as an *Eco*R I-*Hpa* I fragment and ligating it to *Eco*R I-*Eco*R V cut pCR3.

Defective HIV-1 packaging construct Construction of the packaging construct pHyPC required several deletions within the infectious HIV-1 clone pNL4-3 (Adachi *et al.*, 1986) 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 654bp fragment was amplified using primer #1(+)GAAGCGCGCACGGC AAGAGGCGAGGGGCGGCGACTGGTGAGAGATGGGTGCG AGAGCGTCGC, which introduced a 39bp deletion between pos. 749-787 and primers #2(-)GGCCCTGCATGCACTGGATG. The fragment was cleaved with *Bss*H II and *Sph* I. pNL4-3 was cleaved with *Sph* I(pos.1404) and *Eco*R I(pos.5743) and a 4.3kb fragment was isolated. These two fragments (647bp and 4.3kb) were ligated into pHD1, which had been cleaved with *Bss*H II and *Eco*R I. (II) Terminal primers #3:(+)CATAATAAGAATTCTGCAAC and #4:(-) CAAGTTAACAGCACTATTC were used with fusion primers #5:(+)GGGATATTGATGTCTGTAGAATAGGAGCTTTGTTCCT TGGG and #6:(-)CCCAAGGAACAAAGCTCCTATTCTACAG TCATCAATATCCC in order to generate a 1457bp fragment, which contained an 1148bp deletion in the Env region of pNL4-3 (pos.6307-7755). Thus, the fragment was cleaved with *Eco*R II and *Hpa* I. (II) A 240bp fragment, containing the poly(A) site of SV40, was amplified using pJC119 as a template and the following primers: Poly (A) primer 1:(+)TAGCCCGGGATAAGATACATTG ATGAGT; Poly (A) primer 2:(-)TAGGAATTCATCATAATCAGC CATACCAC. This fragment was cleaved with *Sma* I and *Eco*R I. In the final step, the DNA clone from step (I) was cleaved with *Eco*R I, 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.

Generation of defective HIV-1 particles HeLa, HeLaT4, HeLaS2, COS-7, 293, and NIH3T3 were grown to 80% confluence for 24 hours at 37°C on 35 mm dishes in DMEM that contained 10% FBS. The cells were co-transfected with 2.5 μg of pHyPC DNA and 2.5 μg of CD4 DNA using the calcium phosphate precipitation method (Promega). Two days after co-transfection, the medium was collected, clarified by passing through a 0.45 μm filter, and the p24 antigen of the supernatant was quantitated using a HIV-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, pH7.4 (Sigma Chemical Co., St. Louis). CD4 containing particles were precipitated by incubating with 0.5 mg/ml of a polyclonal antibody to CD4 antigen (The National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Program), or with a rabbit anti-CXCR4 antibody (Millennium Biotechnology, CA) at room temperature for 2 hours or overnight at 4°C. The complexes were bound to Protein-A-Sepharose and pelleted by centrifugation (Hong *et al.*, 1999). The particles were disrupted using the lysis buffer from the p24 ELISA kit (Cellular Products Inc.), and the released p24 proteins were quantitated by ELISA.

Centrifugation and fractionation of the defective viral particles Ten milliliter of the medium from the transfected cell was collected and passed through a 0.45 μm filter. Defective particles were concentrated by centrifugation onto a 65% sucrose

pad at 150,000 × g for 60 min. The concentrated particles were resuspended in 0.5 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 h, then fractionated into 0.35 ml aliquots. The concentration of p24 and CD4 proteins was analyzed in parallel by ELISA (Jung *et al.*, 1998).

Results

Incorporation of CD4 into defective HIV-1 particles Using PCR gene fusion techniques, several chimeric CD4 proteins were assembled, as schematized in Figure 1A. Each protein contained 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 (Schubert *et al.*, 1992; Sevier *et al.*, 2000), or the HIV-1 Env protein (Paik *et al.*, 1997; Schubert *et al.*, 1995; Johnson *et al.*, 1998). Two additional CD4 constructs contained the CD4 ectodomain that was fused to the entire gp41 region of HIV-1 Env. The gp41 region had been extended by 20 amino acids into the gp120 region of Env beyond the proteolytic cleavage site at the gp120/gp41 junction. The proteolytic cleavage site was either left unchanged, or it was modified by site-specific mutation.

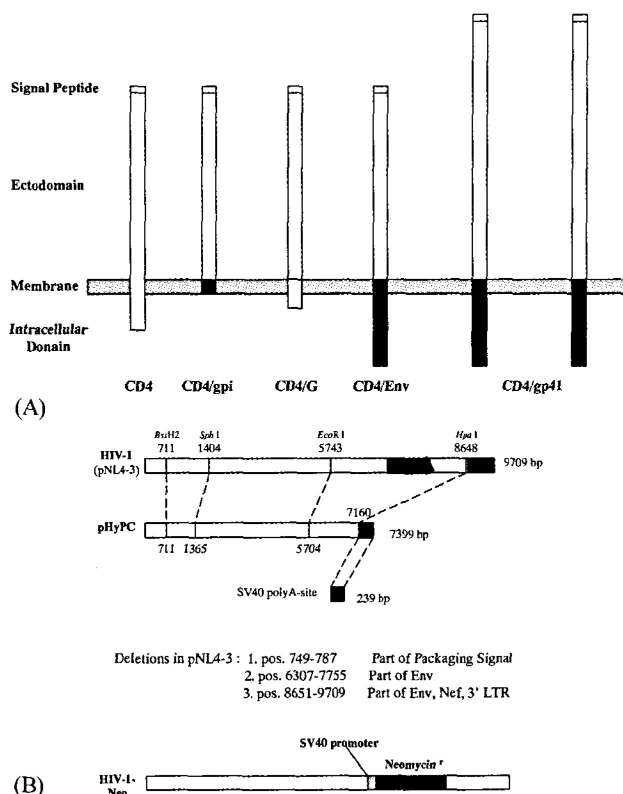

Fig. 1. Chimeric CD4 proteins and defective HIV-1 DNA constructs. The CD4 ectodomain region was precisely fused to different membrane anchors that consisted either of the glycosyl-phosphatidylinositol anchor (CD4/gpi), a HIV-1 gp41 protein with a NH_2 terminal extension of 20 amino acids into the gp120 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 the VSV G protein (CD4/G), respectively. (B) The pHyPC defective HIV-1helper virus construct. Three deletions were made in the infectious DNA clone of HIV-1, pNL4-3. A SV40 polyadenylation site was added. HIV-1-Neo is a defective HIV-1 DNA construct in which part of the Env gene was replaced by a neomycin resistance gene and promoter.

For the generation of defective HIV-1 viruses that carry CD4, we constructed a defective HIV-1 packaging construct, pHyPC (Fig. 1B). 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 of the other sequences, including the genes for Tat and Rev and the RRE, were left intact. This DNA, together with a mini HIV-1 genome that encodes 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.

Co-transfections of pHyPC with the six 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 (Table 1). Between 11% and 45% of the supernatant p24 were precipitated with the anti-CD4 antibody. There was a rough correlation between the amount of CD4 protein that was expressed in the transfected cells and the number of virus particles that could be precipitated. The precipitation of particles that carried 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, which is consistent with the lower CD4/gp41 expression levels. These results suggested that the incorporation of the chimeric CD4 proteins into HIV-like particles seems to correspond more to their expression levels than the type of membrane anchor.

As compared to DNA co-transfections, we obtained even higher yields (over 100 ng/ml) of CD4 bearing virus particles by a 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. A sucrose gradient analysis of the 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

Table 1. Generation of HIV-1 (CD4) Pseudotypes

Pseudotype Virus[a]	P24 (ng)[b]		S/(I+S) (%)	Anti-CD4[c] Precip. (%)
	Interacelluar (I)	Supernatant (S)		
pHyPC	31.1	24.9	44	0
pHyPC (CD4)	24.0	20.5	46	24
pHyPC (CD4/gpi)	19.1	17.1	47	45
pHyPC (CD4/G)	28.2	26.0	48	45
pHyPC (CD4/Env)	22.5	16.8	43	38
pHyPC (CD4/gp41+)	24.0	18.9	44	11
pHyPC (CD4/gp41-)	22.0	21.0	49	16

[a]Pseudotype virus were generated by co-transfection of hela cells with pHyPC DNA and DNAs encoding different CD4 protein chimera.
[b]Two days after transfedction, the amounts of HIV-1 p24 antigen in cell exstracts and cell supernatants were quantified by ELISA.
[c]p24 antigen in cell supernatants was precipitated with anti-CD4 antibodies to estimate the amount of virous carrying CD4.

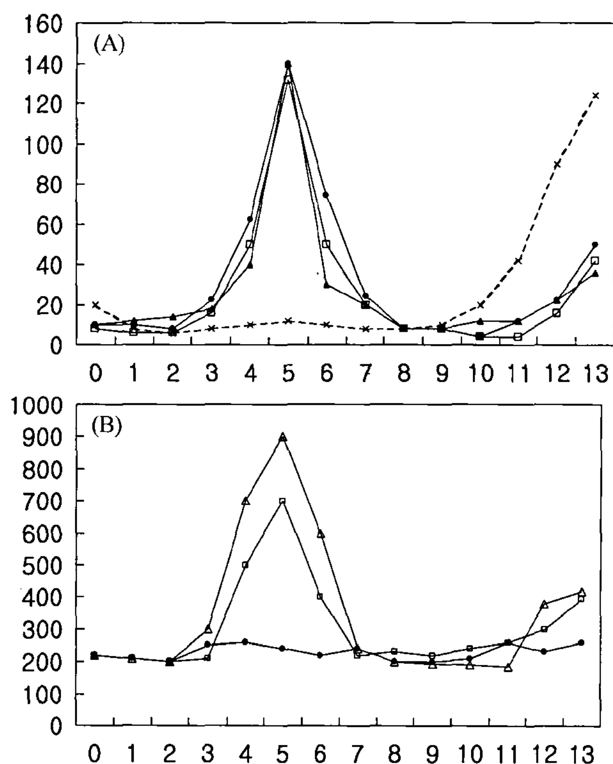

Fig. 2. Separation of pHyPC (CD4) pseudotypes on sucrose gradients. Transfection of HeLa, HeLaT4, or HeLaS2 cells with pHyPC DNA resulted in the release of pHyPC as well as pseudotype pHyPC (CD4) and pHyPC (CD4/gpi) particles, respectively. The particles were concentrated by centrifugation onto a 65% sucrose cushion that was followed by separation through a 15-60% sucrose gradient. Before centrifugation, a portion of the pHyPC particles that were generated in the HeLa cells was pretreated with Triton X-100 to solubilize the viral membrane. The concentration of p24 (Panel A) and CD4 (Panel B) in each gradient fraction was determined by ELISA. The pHyPC particles (●), Triton X-100 treated pHyPC particles (×), pHyPC (CD4) (□), and pHyPC (CD4/gpi) particles (△).

particles (Fig 2A). The position of the p24 peak was the same for the particles that were derived from HeLa, HeLaT4, or HeLaS2 cells.

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 a non-ionic detergent (0.4% Triton) prior to centrifugation. The detergent treatment totally solubilized the envelope of the virus, causing the entire p24 antigen to be shifted to the top of the gradient (Fig. 2A).

To verify that these particles contained CD4 or CD/gpi, the same gradient fractions were also analyzed by ELISA for the presence of the CD4 antigen (Fig. 2B). The peak for the CD4 antigen was also found in gradient fraction No. 5. A calculation of the CD4 to p24 ratio from the same gradient fractions revealed that each HIV-1 particle contained approximately 100 molecules of CD4 or CD4/gpi.

Immunoprecipitation of HIV-1 like particles by anti-CXCR4 antibodies HIV-1 utilizes CD4 as its receptor, but also requires a co-receptor for infections, such as the chemokine receptor, CXCR4, or CCR5 (Choe *et al.*, 1996; Feng *et al.*, 1996; Poles *et al.*, 2001). Since CD4 and modified CD4 proteins were incorporated into HIV-1 like particles, it was of interest to determine whether the co-receptor CXCR4 could also be inserted into the same particles.

The HIV-1 particles were generated by the transfection of pHyPC DNA from human HeLa and 293 cell lines, or from the non-human cell lines, NIH3T3 and COS-7. The particles were compared by precipitation with polyclonal antibodies to CXCR4 protein. As shown in Figure 3, only the CXCR4 antibody precipitated up to 55% of the particles that were generated from the human cell lines. In contrast, less than 7.5% of p24 from the anti-CXCR4 antibodies precipitated

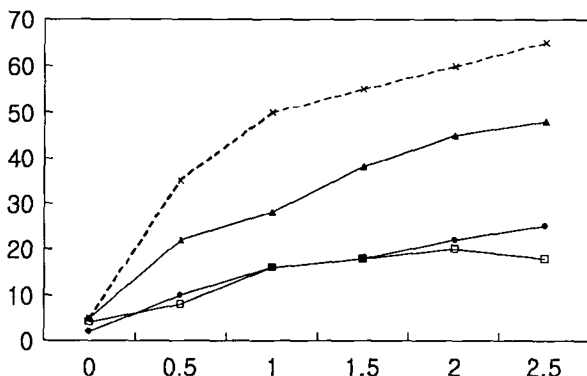

Fig. 3. Immunoprecipitation of pHyPC (CD4) pseudotype virus with anti-CXCR4 antibody. Pseudotype virions were isolated after being co-transfected with animal NIH3T3 (□) and COS-7 (●) cells, or the human 293 kidney (△) and HeLa (×) cells with pHyPC1 and pCR3-CD4 DNA. The amount of immunoprecipitable p24 antigen relative to the amount of input p24 antigen was determined by ELISA and is listed as a percentage.

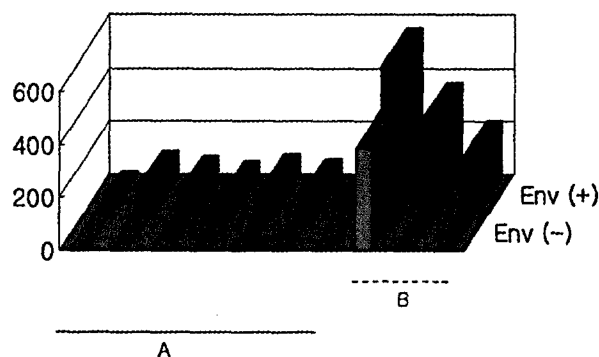

Fig. 4. Stable transformation of Env (+) cells by HIV-1-Neo (CD4/CXCR4) pseudotype virions. Pseudotype HIV-1 particles were isolated from 293 after co-transfection of HIV-1-Neo with the CD4, chimeric CD4, or VSV G protein expression constructs. Some pseudotype viruses were harvested after a single transfection of HIV-1-Neo into the HeLaT4 or HeLaS2 cells that expressed CD4 or CD4/gpi proteins, respectively. An equal number of particles was used to infect the Env (+) TF228 and Env (-) BJAB cells. Stable neomycin resistant transformation colonies were counted after a two-week selection.

transfected NIH3T3 and COS-7 cells. The presence of CXCR4 on HeLa and 293 cells correlates with their ability to form syncytia after the co-expression of CD4 and HIV-1 Env in these cells, which differs from the NIH3T3 and COS-7 cells (data not shown).

The particles that are generated in HeLaT4 and HeLaS2 cells could be precipitated with either anti-CXCR4 or with anti-CD4 antibodies. The amount of anti-CXCR4 antibody perceptible particles that are generated from HeLaT4 and HeLaS2 cells were the same as those generated from HeLa cells. This demonstrates that CXCR4 and CD4 were incorporated into HIV-like particles independently of each other. We can calculate that a minimum of 20%, but more likely all of the particles, contained both CD4 and CXCR4.

Stable transduction of HIV-1 Env expressing cells Pseudotype HIV-1 particles, carrying one of the different chimeric CD4 proteins as well as the co-receptor CXCR4, were isolated after co-transfection of the 293 cells with chimeric CD4 and HIV-1-Neo DNA. Equal amounts of the particles were added to the Env (+) TF228 and Env (-) BJAB cells (Fig. 4). Neomycin resistant colonies were selected in soft agar and quantitated. Surprisingly, all of the chimeric CD4 proteins were functional as HIV-1 receptors when inserted into the viral envelopes, as indicated by the specific and stable transfection of the Env (+) cells. The Env (-) cell were not transduced by the different pseudotype viruses. These results also demonstrated that the amount of the co-receptor CXCR4 that was inserted into the viral envelope from the 293 cells was sufficient and functional during transductions.

In comparison, insertion of the VSV G protein into viral envelopes resulted in a non-selective infection of both the Env (+) and Env (-) cells through endocytosis. The infection levels through the VSV G protein are generally high, but they were only 4-10 fold higher than through direct membrane fusion by CD4-Env. When pseudotype virus particles were generated by single transfections of HIV-1-Neo into the HeLaT4 and HeLaS2 cells, higher levels of the specific infection of Env (+) were achieved (Fig. 4). These results suggest that during co-transfections, the co-expression of CD4 and HIV-1-Neo in the same cell appeared to limit the infectivity of pseudotype particles. The efficient stable transduction by HeLa cells derived HIV-1-Neo (CD4/CXCR4) particles again demonstrates that, similar to the 293 cells, the amount of CXCR4 that is provided by HeLa cells was also sufficient.

Discussion

Targeting the HIV-1 infected cells by pseudotype viruses that carry the HIV-1 receptor CD4 and co-receptor in their envelope is an attractive system to study the pseudotype virus formation and cell targeting. In addition, it is the initial step for the potential development of targeted defective interfering HIV-1 particles. Such particles may be able to exploit wild-type HIV-1 for its proteins, and interfere with its replication while promoting their own replication (Schubert *et al.*, 1995). Such an approach depends on many different elements that need to function together efficiently. This is an interesting challenge that replies on a detailed understanding of all of the HIV-1 replication and pathogenesis. The targeted infection of Env (+) cells can be viewed as one step towards this goal.

We have made several attempts to specifically infect Env (+) cells using CD4 (+)-CXCR4 (+) particles. Infections were followed by the transfer of a neomycin 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 the particles pseudotyped with the VSV G protein, the CD4 (+) and CXCR4 (+) particles were unable to stably transduce the resistance gene (data not shown). The sensitivity of this assay was approximately 1/100 of the virus pseudotyped with the VSV G protein.

The HIV-1 Env protein is not required for the HIV-1 particle formation (Gheysen *et al.*, 1989; Smith *et al.*, 1990; Krausslich *et al.*, 1993). HIV-1 readily forms a pseudotype with other virus envelope protein, for example-the VSV G protein. HIV-1 carrying the VSV G protein can reach levels of viral infectivity that are 100 fold greater than that of the wild-type virus itself (Reiser *et al.*, 1996). In the study presented here, we inserted a modified cellular protein, the HIV-1 receptor, into the envelop of HIV-1 particles. To increase the efficiency of the CD4 protein insertion, we circumvented the HIV-1 induced down regulation of the 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 (Fig. 1A).

In addition, CD4 (+) pseudotype viruses that were generated with VSV and HIV-1 rely on the high level expression of recombinant CD4 and CXCR4. Any targeted virus that is derived from these constructs would be limited by the type of co-receptor that it carries to the target cells that are infected with either a T cell or monocyte/ macrophage tropic strain of HIV-1. For this reason, it was important to see whether basal co-receptor expression levels by the cell would be sufficient for the assembly of a targeted HIV-1 (CD4/ CXCR4) pseudotype.

Cells that express CD4 and HIV-1 Env are able to form large syncytia that involve large membrane segments. The concentration, or the local density of CD4 and CXCR4 in virus particles, may differ. It may not be sufficient to allow fusion of the viral and cellular membranes. Another possibility as to why the infection did not occur may in part involve the newly discovered CD4-dependent Env independent cell binding activity of the CD4 (+) particles. They may block the viral infection.

The fact that CXCR4 was inserted into HIV-1 envelopes at functional levels demonstrated that the high expression levels from recombinant DNA were not required to achieve functional insertion into HIV-1 envelopes. These result are promising. They suggest that, besides CXCR4, other seven-transmembrane proteins, like CCR5, may also be inserted at basal cell expression levels, as compared to higher levels from recombinant DNA. This would allow the generating of both the T-cell tropic and macrophage tropic CD4 (+) pseudotype viruses from the same defective HIV-1 particle.

These results suggest that the CD4 ectodomain may not be fully responsible for this cell binding. However, we cannot completely rule out the possibility that the two different polyclonal antibodies that were used may not have covered all of the regions of CD4 equally well. Attempts to block the potential cellular binding sites by pre-incubating the cells with large amounts of either soluble CD4, or a synthetic CD4 peptide, did not totally abolish the 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 the Env independent ectodomain may not be physically involved in cell binding. Whether the CD4 (+) virus was generated in humans, African green monkeys, Chinese hamsters, or mouse cells, it was not critical for cell binding.

In conclusion, it is encouraging to see how the composition of viral envelopes, like the HIV-1 envelope, can be manipulated so easily through recombinant DNA techniques. The ability to carry out specific targeting of the HIV-1 infected cells, or any cell in the future, remains a challenge that will initially be rewarded with an understanding of the mechanisms of the pseudotype particle formation, viral adsorption, and entry. This understanding will be essential for a future targeted delivery *in vivo* that is safe and efficient.

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