Bimodal down-regulation of CD4 in cells expressing human immunodeficiency virus type 1 Vpu and Env

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We analysed clones of HeLa cells stably expressing the human immunodeficiency virus (HIV-1) envelope gene (env) and the HIV-1 receptor, CD4. Surprisingly, individual clones were found to consist of two distinct populations of cells differing by about 10-fold in the level of surface CD4. When high and low CD4-expressing cells were separated by FACS, each subpopulation gave rise to a mixture of high and low CD4-expressing cells after several days in culture. High and low CD4-expressing subpopulations did not differ with respect to the amount of intracellular Env, but there was an inverse correlation between CD4 and another HIV-1 protein encoded by the same segment of the HIV genome, Vpu. High surface CD4 cells had high levels of intracellular CD4, largely in the perinuclear region, and low levels of Vpu with a diffuse staining pattern. Conversely, low surface CD4 cells had low levels of intracellular CD4 with a diffuse staining pattern, and high levels of Vpu, largely in the perinuclear region. Vectors containing mutant versions of either Env or Vpu failed to down-regulate surface CD4. The phenomenon of bimodal expression of a surface protein in cells derived from single clones provides a simple model of differentiation in vitro. We show how a hypothetical interaction between CD4 and a multimer of Vpu, the multimerization of which is cooperative, would lead to bimodal expression of CD4. This model may be generalized and could explain other cellular 'switches'.

Introduction

Down-regulation of virus receptor in infected cells is a natural phenomenon seen in many retroviruses. In human immunodeficiency virus (HIV)-infected cells, CD4 receptor molecules are depleted from the cell surface possibly because of the formation of intracellular complexes between viral envelope protein (Env) and receptor (Kawamura et al., 1989; Crise et al., 1990; Hart & Cloyd, 1990). The depletion of receptor is thought to be a cause of resistance to superinfection (Rubin, 1960; Vogt & Ishizaki, 1966; Rein, 1982; Granowitz et al., 1991) and natural resistance to retroviral infection in mice and chickens that carry certain endogenous envelope sequences (Gardner et al., 1980; Robinson et al., 1981; Ikeda & Odaka, 1983; Ikeda et al., 1985; Ikeda & Sugimura, 1989; Inaguma et al., 1992; Matano et al., 1993). In addition to Env, HIV encodes two proteins that play additional roles in the down-regulation of CD4: Vpu, an 81 amino acid protein encoded by the bicistronic mRNA that also encodes Env, accelerates degradation of CD4 (Willey et al., 1992; Chen et al., 1993) and Nef, a 27 kDa protein, mediates the internalization and lysosomal degradation of cell surface CD4 (Anderson et al., 1993; Brady et al., 1993).

Vpu-dependent CD4 degradation has been studied in transient transfection experiments using HeLa cells and expression vectors for CD4, Env and Vpu. These transient transfection studies showed that Env binds to CD4 in the endoplasmic reticulum (ER), thereby slowing its transport to the Golgi (Crise et al., 1990; Jabbar & Nayak, 1990), and that Vpu in the presence of Env leads to rapid degradation of CD4 (Willey et al., 1992). Treatment with brefeldin A, which blocks CD4 transport to the Golgi in the absence of Env, also led to rapid degradation of CD4 in the presence of Vpu (Willey et al., 1992), implying that the Vpu effect occurs in a pre-Golgi compartment. The most straightforward model for Vpu-accelerated degradation of CD4 involves Vpu binding to CD4 in the ER. Recently, biochemical evidence has been adduced for a direct physical association between Vpu and CD4 (Bour et al., 1995).

In the transient transfection studies, immunoprecipitation techniques were used to analyse the Vpu-induced changes in the amount of CD4 in lysates of metabolically labelled cells. While providing important information, such studies of whole
cell populations lose information about CD4 expression in individual cells and are subject to artefacts resulting from high levels of protein expression in a small fraction of cells.

To study down-regulation of CD4 in individual cells, we developed a retroviral vector that leads to stable expression of more physiological levels of HIV Env and Vpu in transduced HeLa-CD4 cells (Fujita et al., 1994). We used a mutant env gene (designated 41.2), which encodes an Env protein that binds CD4 but does not form syncytia (Freed et al., 1990), instead of the wild-type env sequence in order to circumvent the problem of fusion between Env-expressing cells and neighbouring CD4+ cells. Immunofluorescence analyses of HeLa-CD4 cell clones expressing Vpu and Env showed unexpected complexity of surface CD4 expression with individual clones being made up of a mixture of high and low CD4-expressing cells, each cell type giving rise to a mixed population of high and low CD4-expressing cells after growth in tissue culture. We describe a model that can explain bimodality of surface CD4 as a result of interaction between CD4 and a multimeric form of Vpu. The model is general in the sense that it shows how genetically identical cells can convert a unimodal distribution of one protein into a bimodal distribution of another protein, a phenomenon that mimics some aspects of differentiation.

Methods

Construction of retroviral vectors. The retroviral vector pKF1 contains a cytomegalovirus (CMV) promoter and HIV rev, vpu and env placed downstream of neo (Fujita et al., 1994). The HIV sequence in pKF1 was derived from the pNL-A3 molecular clone (Adachi et al., 1986). A StuI-BamHI fragment from the env region of pKF1 was replaced with the corresponding restriction fragment from pHenv41.2; this is derived from the BH10 clone of HIV-1, which has a Val to Glu mutation at the second amino acid of gp41 (Freed et al., 1990). The resulting pKF41.2 retroviral vector was used to transduce HeLa-CD4 cells.

Two derivatives of pKF41.2 were constructed with non-functional variants of env or vpu. The env variant contained a mutant env from which the CD4 binding site had been deleted; this mutation leads to production of a stable Env protein that does not bind CD4 (Lasky et al., 1987). This variant was created by replacing the StuI–BamHI fragment of pKF41.2 with the corresponding fragment from clone pNL-A1 (CD4-1) (Willey et al., 1992), resulting in pKF41.2CD4b+. The vpu variant of pKF41.2 was constructed by substituting a segment of vpu containing an 8 bp Xhol linker inserted at an SphI site (position 6155 in pNL4-3). This mutation, originally created in the pNL-A1/U35 clone, leads to a truncated form of Vpu that does not accelerate the degradation of CD4 in a transient expression system (Willey et al., 1992). To transfer this sequence, we ligated SphI-digested pKF41.2 and pNL-A1/U35 and used PCR to amplify a hybrid segment containing the mutation; the hybrid segment contained convenient NolI and DraIII sites flanking the mutation that were used to transfer the mutation to pKF41.2. PCR primers were plus-strand oligo P1 (5' TGTATGGGAATTGGCTCAAAGGAT 3'), which anneals 249 bp upstream of the first codon of rev in pKF41.2, and minus-strand oligo E1 (5' TGATGGAATGCTCAAAAGGAT 3'), which anneals 1141 bp downstream of P1 (position 6681 in pNL4-3).

PCR conditions were 94 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 0.5 min, 30 cycles. The 1141 bp amplified fragment, which contained pKF41.2 sequence upstream of the SphI site (position 5991 in pNL4-3) and pNL-A1/U35 sequence downstream of the SphI site, was digested with NotI (which cuts 81 bp upstream of the first codon of rev in pKF41.2) and DraIII (which cuts at position 6596 in pNL4-3). The 704 bp NotI-DraIII fragment was used to replace the corresponding segment of pKF41.2, yielding pKF41.2/U35.

Tissue culture and retroviral transduction. All cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. HeLa cells and HeLa-CD4 cells were kindly provided by D. Kabat (Oregon Health Sciences University, Portland, Oreg., USA). The HeLa-CD4 clone used in this study was HeLa L cell clone, which expresses a high amount of CD4 (Kabat et al., 1994). Approximately 5 x 10^6 PA317 amphotropic packaging cells (Miller & Buttimore, 1986) were transfected with 5 μg of retroviral vector and 10 μl of Transfectam (Promega) according to the manufacturer’s protocol. Infection of HeLa-CD4 cells and G418 selection were carried out as described previously (Fujita et al., 1994). Individual colonies were isolated and expanded to T25 flasks. Cellular DNA of each clone was extracted using Nucleon (Scotlab) and analysed for the presence of HIV env by PCR with a pair of oligonucleotide primers which anneal to env sequence. Proviral DNAs of clones that were env+ by PCR were analysed by Southern blotting as described previously (Fujita et al., 1994).

Immunostaining. Subconfluent cells grown in T25 flasks were washed with cold PEF (PBS containing 2 mM-EDTA and 5% FCS), collected in 1 ml of PEF and transferred into a 1.5 ml Eppendorf tube. Cells were pelleted by centrifugation for 5 min at 3000 rpm and stained with 50 μl of antibody solution. All antibody solutions were diluted with PEF and incubations were carried out for 1 h on ice. Unbound antibodies were removed by two washes with 1 ml of PEF. Antiserum and dilutions were as follows: unlabeled OKT4 (Ortho diagnostics), 1:5; fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-conjugated OKT4 (Ortho diagnostics), 1:10; P5-3 human monoclonal antibody against Env (NIH AIDS Research and Reference Reagent Program), 1:100; rabbit anti-Vpu antisera (Maldarelli et al., 1993), 1:100; PE-conjugated anti-human IgG and PE-conjugated anti-rabbit IgG (Boehringer), 1:100. For intracellular staining, cells were fixed in 1% paraformaldehyde in PBS (pH 7.5) for 15 min on ice, washed in PEF, stained with antibody solution containing 0.1% saponin, washed with PEF, refixed with 1% paraformaldehyde solution for 15 min on ice and stained with the secondary antibody if necessary. Stained cells were analysed with a Becton Dickinson FACSort, or a Zeiss LSM 410 laser scanning microscope after mounting on a glass slide. For sorting cells, subconfluent cells grown in a T75 flask were removed from the flask with PEF and stained with FITC–OKT4 diluted 1:10 in 0.5 ml of PEF.

Results

Transduction of HeLa-CD4 cells with a retroviral vector expressing fusion-negative HIV-1 Env

Previously, we described a retroviral vector, pKF1, which contains the neo gene driven by a murine retroviralLTR and an internal CMV immediate-early promoter followed by HIV rev, vpu and env placed downstream of the neo gene in the pNL6 retroviral vector (Fujita et al., 1994). We transduced HeLa cells with infectious pKF1 derived from transfected PA317 amphotropic packaging cells and established HeLa cell clones stably expressing high levels of Env. All Env-expressing clones also expressed Rev and Vpu. About 75% of the neo clones did not express Env and contained proviruses that had undergone deletion of env sequence corresponding to a rev intron (Fujita et al., 1994). Although pKF1 gave rise to Env-expressing HeLa...
cells, this vector failed to give Env-expressing clones in HeLa cells expressing CD4. A likely explanation for this failure is that Env-expressing cells fused with neighbouring CD4-expressing cells and died. To avoid possible fusion, we replaced the wild-type HIV-1 rev gene with the 41.2 mutant rev gene, which has a Val to Glu substitution at the second amino acid of gp41. The 41.2 Env is able to bind CD4 but unable to induce fusion with CD4-expressing cells (Freed et al., 1990). PA317 packaging cells were transfected with pKF41.2 and the culture supernatant was used to infect HeLa-CD4 cells. G418-resistant colonies were isolated and their DNAs were analysed for the presence of the rev gene by PCR. HeLa-CD4 clones containing the 41.2 env gene by PCR were further analysed by Southern blotting. Nine of 12 clones analysed contained a single provirus with a unique integration site. Two clones contained proviruses with internal deletions and one clone contained multiple proviruses; these clones were not considered further. One of the nine clones with an apparently intact provirus failed to express Env by Western blot using AIDS patient sera (Fujita et al., 1994). The remaining clones were characterized as described below.

CD4 down-regulation in Env+ HeLa-CD4 cells

To analyse cell surface CD4 expression, env-containing cell clones were stained with FITC-OKT4 and analysed by FACS. In 7/8 clones, two distinct populations of cells, high CD4 and low CD4 expressors, were present within individual clones as shown Fig. 1(a) and Table 1. The amount of surface CD4 on the high CD4 cells was similar to that on untransduced HeLa-CD4 cells. The level of CD4 on the low CD4 cells was 10-fold less than on the parental HeLa-CD4 cells but 3-fold more than the background signal detected on (CD4−) HeLa cells. In each clone, about 20% of the cells were in the low CD4 subpopulation and 80% in the high CD4 subpopulation.

To confirm that the heterogeneity in CD4 levels did not result from inadvertent admixture of clones, we analysed DNA from individual clones for the number of proviral insertions by Southern blot hybridization. A representative experiment is shown in Fig. 2. The HIV env probe detected a 4.6 kb internal EcoRI–BamHI fragment derived from the provirus in all clones with an intact provirus (lanes 2–5). In addition, the probe detected a second fragment that was a different size in each clone, presumably derived from the 3′ virus–cell junction fragment, the length of which is determined by the distance to the nearest EcoRI or BamHI site in the flanking cellular DNA. The presence of a single 'junction fragment' in each clone indicates that the clone is derived from a single proviral integration event and is not a mixture of clones.

To see whether expression of high or low CD4 was a stable characteristic, high and low CD4-expressing cells of the HeLa-CD4 190 clone were separated by FACS. Immediately after separation, the populations were reanalysed by FACS and found to be 98% pure. After 7 days in culture, the 'low' CD4 cells had given rise to a mixture of 53% low and 47% high CD4-expressing cells, whereas the 'high' CD4 cells had given rise to a mixture of 33% low and 67% high CD4-expressing cells (Fig. 1b). Thus, individual cells or their progeny were shifting between two states of CD4 expression.

Analysis of the percentage of cells in different phases of the cell cycle showed no differences between low CD4 cells separated by sorting and the unsorted parental population, indicating that the level of cell surface CD4 was not determined by phase in the cell cycle (data not shown).

Low CD4 cells lack Golgi accumulation of CD4

To analyse intracellular CD4, we blocked staining of...
surface CD4 with unlabelled OKT4, and then permeabilized cells and stained intracellular CD4 with PE-OKT4. The histogram of intracellular CD4 in HeLa-CD4 190 cells showed two peaks with a 4-fold difference in mean fluorescence intensity (Fig. 3a). The intracellular fluorescence of the duller cell population was 10-fold higher than that of (CD4+) HeLa cells used as a negative control. To correlate surface CD4 staining with intracellular CD4, cells were stained for surface CD4 with FITC-OKT4, then stained for intracellular CD4 with PE-OKT4 (Fig. 3c). Low surface CD4 cells contained less intracellular CD4 than high surface CD4 cells. To determine the subcellular localization of CD4, doubly stained cells were analysed by laser scanning confocal microscopy. In cells with bright surface CD4, intracellular CD4 localized mostly in the perinuclear area, which includes the Golgi apparatus. In contrast, in cells with low surface CD4, the intracellular CD4
**Bimodal CD4 down-regulation by HIV Vpu and Env**

![Image of FACS analysis of surface and intracellular CD4](image-url)

**(a) Intracellular CD4 expression**

![Graph showing intracellular CD4 expression](graph-url)

**(b) Surface CD4 expression**

![Graph showing surface CD4 expression](graph-url)

**(c) Differential staining of surface and intracellular CD4**

![Graph showing differential staining](graph-url)

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Fig. 3. FACS analysis of surface and intracellular CD4. (a) Intracellular CD4 staining. Cells were incubated with unlabelled OKT4 for 1 h to block surface CD4. These cells were fixed with 1% paraformaldehyde and stained with PE-OKT4 in the presence of 0.1% saponin. (b) Surface CD4 staining. Cells were stained with FITC-OKT4. In (c) and (b), the left-hand panels are an overlay of histograms for HeLa cells (shaded) and HeLa-CD4 cells, whereas the right-hand panels are histograms of the single Rev+Vpu+Env+ HeLa-CD4 clone 190. (c) Differential staining of surface and intracellular CD4. Cells were stained for surface CD4 with FITC-OKT4 and fixed in paraformaldehyde. The fixed cells were then stained with PE-OKT4 in the presence of saponin. The sensitivity settings of the flow cytometer were not the same as in the experiment shown in Fig. 1; hence slight differences in the x-axis positions of identical cell types are not unexpected.

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was more homogeneously distributed throughout the cytoplasm (Fig. 4). Since CD4 is known to transit from ER to the Golgi apparatus, these results are consistent with reduced transport of CD4 to the Golgi in cells with low surface CD4.

**Low CD4 cells express a high amount of Vpu**

HeLa-CD4 190 cells expressed both Env and Vpu as shown by the histograms in Fig. 5. To look for a possible correlation between surface CD4 and expression of Env or Vpu, cells were stained for surface CD4 with FITC-OKT4, then fixed with paraformaldehyde followed by intracellular staining with anti-Vpu or anti-Env. The intracellular signals were visualized with PE-conjugated secondary antibodies that do not cross-react with OKT4 (see Methods). High and low surface CD4 cells stained equally with an anti-Env monoclonal antibody (Fig. 5a, c). In contrast, low surface CD4 cells expressed only high amounts of Vpu, whereas high CD4 cells were quite heterogeneous for expression of Vpu (Fig. 5d, f). When examined by confocal microscopy, low surface CD4 cells (Fig. 6a) showed perinuclear concentration of Vpu that was more prominent than in high surface CD4 cells (Fig. 6b). These results indicate that the subpopulations of high and low surface...
Fig. 4. Difference in the intracellular localization of CD4 in high and low CD4 cells. HeLa-CD4 190 cells were sequentially stained with FITC-OKT4 (surface CD4) and PE-OKT4 (intracellular CD4) as described in Fig. 3 (c). Low surface CD4 cells are indicated by arrows.

Fig. 6. Difference in Vpu expression in high and low surface CD4 cells. HeLa-CD4 190 cells were stained for surface CD4 with FITC-OKT4 and intracellular Vpu with anti-Vpu antibody followed by PE-conjugated secondary antibody as described in Fig. 5. (a) Low surface CD4 cells; (b) high surface CD4 cells.

CD4 cells did not differ in the amount of Env but did differ in the amount and apparent location of Vpu.

Retroviral vectors encoding defective Vpu or a mutant Env that is unable to bind CD4 do not decrease surface CD4

It was shown previously that transient expression of Vpu + Env enhances the rate of degradation of CD4 in the ER of HeLa-CD4 cells (Willey et al., 1992). In the transient expression system Vpu-induced CD4 degradation was not seen when an Env mutant lacking the CD4 binding region was expressed instead of wild-type Env, suggesting that interaction between Env and CD4 was necessary for Vpu-induced degradation of CD4. To see if the down-regulation of surface CD4 in stably transduced cells was also due to a combined effect of Vpu and Env, we made two derivatives of pKF41.2 containing mutant env or vpu. pKF41CD4bs contains wild-type vpu but its env is deleted for the CD4 binding site, whereas pKF41.2/U35 contains the 41.2 env with a mutant, truncated vpu which does not accelerate CD4 degradation in the transient system (see Methods; Willey et al., 1992). HeLa-CD4 cells were stably transduced with these retroviral vectors and analysed for surface CD4 expression. As summarized in Table...
Vpu and Env is responsible for surface CD4 down-regulation with decreased CD4 expression. Thus, the combined action of required expression of both Vpu and Env (Table 1). Down-regulation of CD4 in our system by the fact that the Golgi-like distribution of Vpu (Willey et al., 1994) and is suggested in our system, the fact that our cells are bimodal with respect to expression of Vpu, intracellular CD4 was lower in low surface CD4 cells than in high surface CD4 cells (Fig. 3c, far-right panel) is consistent with rapid intracellular degradation.

Perhaps our most interesting finding was that cells derived from individual clones were bimodal with respect to the amount of CD4 on the cell surface - some cells had the same amount as parental HeLa-CD4 cells and others had 10- to 20-fold less (Figs I and 3). Low and high CD4 cells each gave rise to mixtures of low and high CD4 cells after several days in tissue culture. This system has features of a reversible cellular switch in which genetically identical cells 'choose' one of two states - low or high surface CD4 - which are stable over short periods of time but interconvert in such a way that the population as a whole tends toward a fixed ratio of numbers of cells in the two states.

A similar bimodal down-regulation of CD4 was recently reported in clones of U937 cells transfected with an expression vector encoding HIV-1 Rev, Vpu and Env under an inducible metallothionein II A promoter (Koga et al., 1994). When the promoter was induced with CdCl\textsubscript{2}, Env expression was enhanced and nearly complete down-regulation of surface CD4 was seen in almost all cells (Fig. 3 of Koga et al., 1994). In cells not treated with CdCl\textsubscript{2}, less Env was expressed (Fig. 2 of Koga et al., 1994), and FACS analysis of these cells stained for surface CD4 with OKT4 showed two populations of cells (Fig. 3 of Koga et al., 1994), as seen in our system. Although expression of Vpu was not analysed in the U937 clones, we found that down-regulation of CD4 correlated with the amount of Vpu but not the amount of Env (Fig. 5).

How can bimodality be explained? If down-regulation of CD4 is a consequence of CD4 interacting with Vpu, it could be that our cells are bimodal with respect to expression of Vpu.
with some cells failing to express Vpu at all or having Vpu levels below a threshold required for CD4 down-regulation. Because the signal-to-noise ratio of our anti-Vpu antiserum was low, there was considerable overlap between Vpu-containing HeLa-CD4 190 cells and Vpu-negative HeLa-CD4 cells (Fig. 5c). It is therefore difficult to say whether the bulk of high surface CD4 cells have no Vpu or just very low levels of Vpu.

A second possibility is that our HeLa-Rev+Vpu+Env+ clones are unimodal with respect to expression of Vpu, but that the relationship between Vpu and CD4 is non-linear with slight increases in Vpu leading to a dramatic fall in CD4. Thus an increase in Vpu from about 50 fluorescence units (FU) to 100 FU was associated with more than a 10-fold reduction in surface CD4 (Fig. 5d). Non-linearity suggests a cooperative phenomenon involving Vpu and CD4. Vpu has been reported to multimerize (Maldarelli et al., 1993), and protein multimerization is often a cooperative phenomenon. Our observations would be largely explained if CD4 formed a complex with multimeric Vpu, the multimerization of which was a cooperative phenomenon, and if the complex of CD4 and multimerized Vpu was rapidly degraded before transport through the Golgi to the cell surface. There is evidence that CD4 forms a complex with Vpu (Bour et al., 1995), but no evidence as yet that Vpu is multimerized in the complex.

Despite the general inverse correlation between Vpu and surface CD4, it is clear from Fig. 5(f) that some cells with Vpu above background have high surface CD4. These cells could be in transit between high and low surface CD4 states. We recently found that when such clones are treated with brefeldin A, an agent that accelerates the Vpu-dependent degradation of CD4 (Willey et al., 1992), intracellular CD4 in some of the high surface CD4 cells falls rapidly, indicating Vpu-dependent degradation of CD4 in these cells. This suggests that brefeldin A induces a transition of some high surface CD4 cells to the low surface CD4 state. These transitions mimic the conversion of FACS-separated high surface CD4 cells to low surface CD4 cells after several days in culture (Fig. 1b).

We believe that the phenomenon of bimodal expression of proteins in cells derived from single clones is not uncommon. Koga et al. (1994) observed bimodal expression of CD4. Masuda et al. (1990) reported a neo-selected clone of mouse cells that was bimodal for expression of murine retroviral Env, and we have observed bimodal expression of the ecotropic murine leukemia virus receptor in CHO cells (unpublished). Bimodality could result from down-regulation or rapid degradation of the protein in question in cells expressing more than a critical level of an interacting protein. Such a mechanism would lead to bimodality even if the interacting protein had a bell-shaped distribution among genetically identical cells. Unknown stochastic or physiological variables that altered levels of the interacting protein could then lead to transitions between two ‘states’ defined by the level of the first protein, constituting a kind of cellular ‘switch’.

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