Human Immunodeficiency Virus Type-1 Susceptible Whole Cell and Microcell Hybrids*†

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ABSTRACT

Expression of CD4, the principle receptor for HIV-1, is not sufficient for viral entry into most non-human and rare human cell lines. Construction of HIV-1 susceptible heterokaryons and a hybrid cone by fusion of HeLa cells and the HIV-1 resistant human cell line U373-CD4 were previously reported by us. These results suggested that U373-CD4 lack a cofactor(s) which is essential for HIV-1 entry and can be supplied by HeLa cells. Now the construction of multiple stable U373-CD4/HeLa whole cell and microcell hybrid clones are described, two of which are highly susceptible to HIV-1. Using these hybrids it is demonstrated that expression of CD4 and CD26, the T cell activation antigen dipeptidyl peptidase IV recently proposed as a CD4 cofactor necessary for HIV-1 infection, are not sufficient for HIV-1 entry. This panel of HIV-1 resistant and susceptible hybrids provides a rapid, simple assay for testing the role of other candidate cofactor molecules (e.g., fusin) that may be required for HIV-1 entry into human cells. Further, the method described by us for constructing HeLa microcells should permit the construction of murine-HeLa microcell hybrids, thus providing ideal reagents for determining which human chromosome(s) are needed to confer HIV-1 susceptibility onto non-human cells.

Introduction

The infection of most cells by the human immunodeficiency virus type 1 (HIV-1) is mediated by CD4, the principal cellular receptor for HIV-1.1,2 Viral entry is accomplished in a stepwise manner that begins with the high-affinity binding of the HIV-1 envelope protein gp120 to CD4 and ends with virus-to-cell membrane fusion which is mediated by the hydrophobic N-terminus of the viral subunit protein gp41.3,4,5 Independent of the initial binding to CD4, the V3 loop of gp120 plays a key role in viral entry6,7
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and cell tropism and has several proteolytic cleavage sites which has led to the suggestion that cell surface proteases may play a role in HIV-1 entry and syncytia formation, as is the case with other retroviruses.  

While essential for efficient HIV-1 infection, the expression of CD4 is not sufficient for viral entry into most non-human cell lines as well as rare human cell lines. Gp120 binds to CD4 on these resistant cells; however, viral entry does not occur, and syncytia do not form when these cells are co-cultured with HIV-1 envelope-expressing cells. Furthermore, vesicular stomatitis virus pseudotyped with the HIV-1 envelope protein cannot infect these cells, supporting the notion that the block to infection is at the level of viral entry. One interpretation of these data is that CD4+ HIV-1 resistant cells lack a cofactor necessary for virus-to-cell membrane fusion. Consistent with this hypothesis, it has been shown by us and others that HeLa cells can complement the entry defect of CD4+ resistant cells when used as partners in heterokaryon formation.  

Furthermore, a hybrid cell line was established from an HIV-1 resistant human glioblastoma cell line (U373-CD4) and HeLa cells, which was initially permissive for HIV-1 but became refractory to infection in subsequent cell passages. Two other groups have reported the construction of stable murine-human somatic whole cell hybrids which are permissive for HIV-1 infection. A number of cellular proteins including LFA-1, CD7 and several uncharacterized molecules have been reported to be important for CD4 mediated HIV-1 infection, but none are absolutely necessary for infection in all cell types. The T-cell activation antigen dipeptidyl peptidase IV, CD26, has been proposed as a necessary cofactor for HIV-1 entry into CD4+ mouse cells, however, subsequent studies of both human and non-human cells did not confirm this finding. Recently, a putative G protein-coupled receptor designated "fusin" has been shown to permit HIV-1 infection of CD4-expressing non-human cells and to promote fusion between HIV-1 envelope (Env)-expressing cells and a variety of CD4-expressing, HIV-1 resistant human and non-human cell lines. However, fusin did not promote fusion of cells when Env molecules were derived from macrophage tropic HIV-1 isolates.  

A set of stable somatic whole cell and microcell hybrid clones was constructed by fusing 2 HIV-1 resistant human cell lines. Two clones from this set are highly susceptible to HIV-1 infection. Using the parental cell lines and HIV-1 susceptible and resistant clones, it has been demonstrated that there is no correlation between CD26 expression and susceptibility to HIV-1.  

Materials and Methods

CELL LINES

All cell lines were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin, streptomycin, glutamine and 10% Nu-serum.* U373-CD4-LTR/β gal cells are an HIV-1 resistant human glioblastoma clone, derived from U373-MG cells, which expresses CD4 and has been stably transfected with the β-galactosidase (β-gal) gene under the control of a truncated HIV-1 long terminal repeat (HIV-LTR). Expression of tat in these cells activates transcription from the LTR, resulting in β-gal activity, which can be assayed by 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) staining. HeLa-CD4-LTR/β-gal cells† are an

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HIV-1 susceptible cell line which expresses CD4 and contains a single integrated copy of the β-gal gene driven by the HIV-LTR. HeLa-puro cells, a HeLa cell line containing chromosomes randomly tagged with the puromycin resistance gene puro, were constructed by transduction of HeLa cells with an amphotropic retroviral vector generated by calcium phosphate transfection of pBabePuro into the packaging line PA317.

**Viruses and Infections**

HIV-1 (laboratory-adapted isolate (LAI)) and L tatSN, an amphotropic retroviral vector encoding *tat*.*† HIV-1 infections were performed in the presence of DEAE-dextran (20 μg/ml) at a multiplicity of infection (moi) of 0.1 to 0.2 MAGI units/cell, and L tatSN infections were performed at a moi at 0.25 MAGI units/cell with polybrene at a concentration of 4 μg/ml.

X-gal staining for β-gal activity, performed at 5 to 6 days post infection for HIV-1 and 3 days post infection for L tatSN, is described elsewhere.*35

**Cell and Microcell Hybrid Construction**

Whole cell hybrids were generated by plating equal numbers of U373-CD4-LTR/β-gal cells and HeLa-puro cells onto wells of 6 well plates to obtain subconfluent monolayers. The following day, cells were washed three times with serum-free DMEM and overlaid with 1 ml of prewarmed polyethylene glycol (PEG, molecular weight, 1,500)§ in serum-free medium (50%, wt/vol). After 1 min the PEG was removed, and the cells were washed three times with serum-free DMEM and fed with DMEM plus 10% Nu-serum. The next day G418 (800 μg/ml, active weight), hygromycin (100 μg/ml) and puromycin (1 μg/ml) were added to select hybrid clones which were later isolated with cloning rings.

Microcell hybrids, which generally have simpler karyotypes than whole cell hybrids, were made by fusing HeLa-puro microcells to U373-CD4-LTR/β-gal cells. HeLa-puro microcells were generated by including HeLa-puro cells to micronucleate with prolonged exposure to colcemid (55 to 72 hrs at 0.05 μg/ml) followed by centrifugation, in the presence of cytochalasin B (10 μg/ml), either through a percoll density gradient or from concanavalin A coated plastic bullets.*40 Microcell preparations were then purified by serial filtration through 8 and 5 μm membranes, seeded onto 25 cm flasks of U373-CD4-LTR/β-gal monolayers in serum free DMEM containing phytohemagglutinin P (200 μg/ml) and incubated at 37°C for 20 min. PEG-mediated fusion, hybrid selection, and clone isolation were performed as described previously for whole cell hybrids.

**Fluorescence-Activated Cell Sorter Analysis**

Fluorescence-activated cell sorter (FACS) analysis of cell lines was performed as previously described.*16 CD26 expression was assayed using the fluorescein isothiocyanate (FITC)-conjugated anti-CD26 antibody Ta1-FITC.* The IgG1 anti-mouse-CD31 antibody 31.A† followed by a FITC-conjugated goat-anti-mouse secondary antibody‡ was used as an isotyped-matched negative control for each cell line.

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Northern Blot Analysis

Northern blot hybridization using a CD26 specific probe constructed from the plasmid pSRα-CD26.241§ was performed as described previously.42

Results

HIV-1 Infection of U373-CD4-LTR/β-gal/HeLa Hybrids

Since an HIV-1 susceptible (but unstable) cell hybrid had been previously constructed from the two HIV-1 resistant human cell lines U373-CD4 and HeLa,16 a large series of stable whole cell and microcell hybrids were sought from these same parental cell lines with the intent of identifying the HeLa chromosome(s) which can complement the resistance of U373-CD4 cells to HIV-1 infection. To provide a simple assay for HIV-1 infection of complemented U373-CD4/HeLa hybrids, the indicator cell line, U373-CD4-LTR/β-gal was used as fusion partners for puromycin resistant HeLa cells and microcells. HeLa-puro microcells were used to permit the transfer of one or few HeLa chromosomes yielding hybrids with simpler karyotypes than those of whole cell hybrids.40 A total of 45 U373-CD4-LTR/β-gal/HeLa hybrid clones (27 whole cell and 18 microcell) were isolated and were tested for susceptibility to HIV-1 by infecting them with HIV-1 (LAI strain) and assaying for Tat-induced β-gal expression. Two clones (whole cell hybrid 6 and microcell hybrid 9m) were highly susceptible to HIV-1 yielding thousands of blue cells/22 mm-diameter well after HIV-1 infection and X-gal staining (figure 1). Thirty-six of the remaining 43 hybrid clones were minimally susceptible to HIV-1 yielding between 5 and 20 blue cells/well, and 7

were not susceptible yielding only background β-gal expression (5 or fewer blue cells/well) after HIV-1 exposure. The X-gal staining patterns of 2 of the 7 resistant clones (whole cell hybrid 43 and microcell hybrid 2m) after incubation with HIV-1 are shown in figure 1.

As shown previously, HeLa-CD4 cells but not U373-CD4 cells were susceptible to HIV-1. All of the cell lines contained HIV-LTR/β-gal reporter constructs as demonstrated by β-gal expression after infection with the tat-expressing amphotropic retroviral vector LtatSN (figure 1, right column) and little or no background β-gal expression in mock infected cells (figure 1, middle column). Notably, later passages of hybrid clones 6 and 9m remained highly susceptible to HIV-1.

CD26 Expression in Hybrid and Parental Cell Lines

To determine whether or not the viral susceptibility of the U373-CD4-LTR/β-gal/HeLa clones and their parental cells correlated their expression of CD26, the 2 highly susceptible clones (6 and 9m), 2 of the 7 resistant clones (43 and 2m), and the parental cells for the presence of CD26 were tested by FACS analysis. In figure 2a it is demonstrated that all 4 U373-CD4-LTR/β-gal/HeLa hybrid clones, U373-CD4-LTR/β-gal cells, and HeLa-CD4-LTR/β-gal cells expressed CD26 regardless of their susceptibility to HIV-1. To corroborate our FACS data demonstrating that CD26 is expressed in both the HIV-1 susceptible and resistant hybrid clones as well as in the parental cells, a northern blot analysis was performed of all the cells using a CD26 specific probe. In figure 2b it is shown that the 3.9-kb major CD26 transcript is expressed at similar abundance in all 4 hybrids and the parental cells. These findings support our FACS results demonstrating CD26 expression in the cell hybrids and their parental cells.

§ Provided by C. Morimoto, Dana Farber Cancer Institute, Boston, MA.
Figure 1. Human immunodeficiency virus-1 (HIV-1) infection of cell hybrids and parental cells. Sub-confluent monolayers of cells in 12 well plates were incubated with HIV-1 (LAI strain) (left column), LtatSN (right column) or were mock infected (middle column) before assaying for β-gal expression as described in Materials and Methods. Whole cell hybrid 6, microcell hybrid 9m and HeLa-CD4-LTR/β-gal cells were highly susceptible to HIV-1 yielding thousands of blue cells per well while hybrid clones 43 and 2m and U373-CD4-LTR/β-gal cells were resistant to HIV-1. All cells contained Tat-inducible LTR/β-gal constructs as demonstrated by infection with LtatSN (right column). Background β-gal expression was minimal in all cell lines (middle column). Magnification is ×100.

Discussion

This is the first report describing the construction of stable, HIV-1 susceptible whole cell and microcell hybrids from 2 HIV-1 resistant human cell lines. It has previously been reported by us and others that HeLa cells, when used as fusion partners in transient heterokaryon formation with a variety of HIV-1 resistant, CD4-expressing human and non-human cells, can supply the cofactor(s) needed for HIV-1 entry.15,16,17,43 Additionally, a recent report described heterokaryons formed from proteinase treated human erythrocyte ghosts and CD4+ mouse
FIGURE 2. CD26 surface expression of cell hybrids and parental cells. Cells were analyzed for CD26 expression by fluorescence-activated cell sorter directly with the fluorescein isothiocyanate conjugated anti-CD26 antibody Tal (thick lines) and indirectly with the isotype-matched negative control anti-mouse-CD31 antibody 31.A (thin lines).

FIGURE 2B. Northern blot analysis of CD26 expression in cell hybrids and parental cells. Total cellular RNA (3 μg/lane) from hybrid clones 6, 9m, 43, and 2m and HeLa-CD4-LTR/β-gal cells (HeLa/β-gal) and U373-CD4-LTR/β-gal cells (U373/β-gal) was analyzed for the presence of CD26 transcripts by hybridizing with a 32P-labeled CD26 specific probe. Similar levels of the 3.9 kb major CD26 transcript were detected in all cell lines.
cells which allow HIV-1 envelope/CD4-mediated fusion.\textsuperscript{44} Previously, a single U373-CD4/HeLa hybrid clone was described by us which was initially permissive for HIV-1 but became resistant to infection in subsequent cell passages.\textsuperscript{16}

Two other groups have reported establishing murine-human hybrids which were permissive for HIV-1.\textsuperscript{18,19} Karyotypic analysis of the 3 interspecies hybrids formed in one of these studies\textsuperscript{19} suggested a correlation between the absence of human chromosomes 1, 3 and 9 and the failure to allow full viral replication. However, all the hybrids in that study may have permitted some degree of viral replication as assayed by electron microscopy, and none appeared to hinder viral entry which is thought to be the major block to HIV-1 infection of murine cells. Furthermore, since all 3 hybrids had different karyotypes and all contained at least 13 human chromosomes, it was not possible to identify the human chromosome(s) needed (besides chromosome 12 which encodes CD4) for CD4-mediated virus-to-cell membrane fusion and HIV-1 entry.

Microcell fusion is a technique used to transfer one or few chromosomes (or chromosome fragments) from a donor to a recipient cell line to generate cell hybrids with relatively simple karyotypes. Though the generation of HeLa microcells has not, to our knowledge, been reported previously, conditions of prolonged, low concentration colcemid arrest were discovered which enabled generation of fusion-competent, biologically active HeLa microcells. Our finding that HeLa microcells can complement the HIV-1 entry defect of U373-CD4-LTR/β-gal cells suggests that it may be possible to map the complementing CD4 cofactor(s) to one or few chromosomes.

Since both parental cell lines used in our study were human and polyploid, it would be difficult to identify which hybrid cell chromosomes are of HeLa origin other than those tagged with the puromycin resistance gene. However, since heterokaryons formed from whole cell fusions of HeLa cells to CD4+ murine cells permit HIV-1 entry,\textsuperscript{15,17} and since stable, HIV-1 susceptible human-murine hybrids have been described, it should be possible to establish HeLa microcell-murine hybrids which allow HIV-1 infection. The HeLa chromosome(s) present in these interspecies microcell hybrids could then be easily identified and the chromosome(s) encoding the CD4 cofactor(s) needed for HIV-1 infection determined.

Since CD26 had recently been proposed as a cofactor in HIV-1 entry\textsuperscript{25} it was tried to determined whether or not CD26 expression in our parental cell lines, whole cell hybrids and microcell hybrids correlated with their susceptibility to HIV-1. Our finding that both HIV-1 resistant parental cell lines as well as HIV-1 resistant (43 and 2m) and susceptible hybrids (6 and 9m) expressed similar levels of CD26 mRNA and protein suggests that CD26 expression in addition to CD4, is not sufficient for HIV-1 infection. No test was run to determine whether or not CD26 is enzymatically active in each cell line; however, the original report which proposed CD26 as a cofactor for HIV-1 entry suggested that CD26 might function in HIV-1 entry independent of its peptidase activity.\textsuperscript{25} Our data are consistent with those of a number of reports published since the 1993 article by Callebaut et al.\textsuperscript{25} which have failed to support a role for CD26 in HIV-1 cell entry.\textsuperscript{26,27,28,29,30,31,32,33}

The recent identification of fusin as an entry cofactor for lymphocyte tropic strains of HIV-1\textsuperscript{34} provides another candidate cofactor molecule which can be easily tested in our system. Furthermore, since fusin does not function as an entry cofactor for macrophage tropic HIV-1 iso-
lates, our cell lines could be used to help identify fusin-like molecules used during infection with macrophage tropic and primary HIV-1 strains.

In summary, the construction of stable HIV-1 susceptible whole cell and microcell hybrids from 2 HIV-1 resistant human cell lines are described. Our success in generating functional HeLa microcells makes possible the construction of murine-HeLa microcell hybrids which might be infectible with HIV-1 and thus provide an ideal system for identifying the human chromosome(s) needed for CD4-mediated HIV-1 infection.

Additionally, our panel of HIV-1 susceptible and resistant U373-CD4-LTR/β-gal/HeLa hybrids allowed us to demonstrate that CD26 and CD4 expression alone are insufficient for HIV-1 entry and provides a rapid simple assay for testing other candidate cofactor molecules like fusin. Finally, the reagents described in this report can be used to help identify the CD4 cofactors necessary for infection with HIV-1 isolates having varied (non-lymphocytic) cell tropisms. The discovery of the cofactor molecules needed for HIV-1 entry may lead to the development of anti-HIV-1 therapies which target this early step in the viral life cycle.

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