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Cell Type-Specific Fusion Cofactors Determine Human Immunodeficiency Virus Type 1 Tropism for T-Cell Lines versus Primary Macrophages

GHALIB ALKHATIB, CHRISTOPHER C. BRODER, AND EDWARD A. BERGER*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

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Work in this laboratory previously demonstrated that the tropism of different human immunodeficiency type 1 isolates for infection of human CD4$^+$ continuous cell lines (e.g., T-cell lines and HeLa-CD4 transformants) versus primary macrophages is associated with parallel intrinsic fusogenic specificities of the corresponding envelope glycoproteins (Envs). For T-cell line-tropic isolates, it is well established that the target cell must also contain a human-specific fusion cofactor(s) whose identity is unknown. In this study, we tested the hypothesis that the Env fusion specificities underlying T-cell line versus macrophage tropism are determined by distinct cell type-specific fusion cofactors. We applied a recombinant vaccinia virus-based reporter gene assay for Env-CD4-mediated cell fusion; the LAV and Ba-L Envs served as prototypes for T-cell line-tropic and macrophage-tropic isolates, respectively. We examined CD4$^+$ promyeloctic and monocytic cell lines that are infectible by T-cell line-tropic isolates and become susceptible to macrophage-tropic strains only after treatment with differentiating agents. We observed parallel changes in fusion specificity: untreated cells supported fusion by the LAV but not the Ba-L Env, whereas cells treated with differentiating agents acquired fusion competence for Ba-L. These results suggest that in untreated cells, the block to infection by macrophage-tropic isolates is at the level of membrane fusion; furthermore, the differential regulation of fusion permissiveness for the two classes of Envs is consistent with the existence of distinct fusion cofactors. To test this notion directly, we conducted experiments with transient cell hybrids formed between CD4-expressing nonhuman cells (murine NIH 3T3) and different human cell types. Hybrids formed with HeLa cells supported fusion by the LAV Env but not by the Ba-L Env, whereas hybrids formed with primary macrophages showed the opposite specificity; hybrids formed between HeLa cells and macrophages supported fusion by both Envs. These results suggest the existence of cell type-specific fusion cofactors selective for each type of Env, rather than fusion inhibitors for discordant Env-cell combinations. Finally, analyses based on recombinant protein expression and antibody blocking did not support the proposals by others that the CD44 or CD26 antigens are involved directly in the entry of macrophage-tropic isolates.

Individual isolates of human immunodeficiency virus type 1 (HIV-1) show markedly distinct tropisms for infection of different CD4$^+$ cell types in vitro (reviewed in references 34, 42, and 51). Most isolates obtained directly from infected individuals (particularly during the asymptomatic phase) replicate efficiently in primary macrophages but poorly in CD4$^+$ continuous cell lines (e.g., T-cell lines and HeLa-CD4 transformants); these strains are designated macrophage tropic. Other isolates (generally obtained from patients during the symptomatic phase and also selected after long-term virus propagation in T-cell lines) show the opposite selectivity, replicating more efficiently in CD4$^+$ continuous cell lines than in primary macrophages; such strains are designated T-cell line tropic. These distinct cytotropisms, although not absolute (14, 52), have profound implications for disease progression (34) and for transmission of the infection between individuals (16).

The primary viral determinants for T-cell line versus macrophage tropism of HIV-1 reside in the envelope glycoprotein (Env), particularly in regions of the external subunit (gp120) containing the third hypervariable loop (V3) (11–13, 28, 39, 48, 54). A similar situation holds for the related simian immunodeficiency virus (26, 29, 35). These findings raise the possibility that the principal restriction between a viral isolate and a nonpermissive CD4$^+$ cell type is at the level of the membrane fusion reactions involved in virion-cell entry and the related process of syncytium formation (cell fusion). Although contrary arguments have been put forth for both HIV-1 (27, 43, 46) and simian immunodeficiency virus (35), most experimental evidence supports this notion (4, 10, 12, 24, 29, 33, 37, 39, 47). In particular, we recently used a recombinant vaccinia virus expression and assay system (37) to demonstrate a marked parallel between the intrinsic fusogenic selectivities of various HIV-1 Envs and the infection cytotropisms of the isolates from which they were derived (4).

One model to account for this fusion selectivity is based on studies using stable or transient cell hybrids (5, 17–19, 25, 44, 53). Experiments with T-cell line-tropic isolates indicated that the Env-CD4 interaction results in membrane fusion only if the CD4$^+$ target cell contains an essential fusion cofactor(s). The cofactor has yet to be identified, although functional studies suggest that it is present on diverse human cell types but absent from nonhuman cells (3, 15, 32). In this study, we investigated the hypothesis that T-cell line versus macrophage tropism of different HIV-1 isolates results from the requirement of the

* Corresponding author. Mailing address: Laboratory of Viral Diseases, NIAID, National Institutes of Health, Building 4, Room 236, Bethesda, MD 20892. Phone: (301) 402-2481. Fax: (301) 480-1147. Electronic mail address: edward_berger@nih.gov.

corresponding Envs for distinct fusion cofactors that are differentially expressed on various $CD4^+$ target cell types.

MATERIALS AND METHODS

Cells and culture conditions. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Continuous cell lines were obtained from the American Type Culture Collection, Rockville, Md. The HeLa and NIH 3T3 cell lines were maintained in DMEM-10 (Dulbecco's modified Eagle's medium [Quality Biologicals, Gaithersburg, Md.] supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics). The Jurkat T-cell line and the promyelocytic HL-60 cell line were maintained in IMDM-20 (Iscove's modified Dulbecco's medium [GibcoBRL] supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and antibiotics). The promonocytic THP-1 cell line was maintained in RPMI-10 (RPMI 1640 medium [Quality Biologicals] supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], and antibiotics). Where indicated, HL-60 and THP-1 cells (5×10^5/ml) were cultured for 5 days with 1 μM all *trans*-retinoic acid (Sigma, St. Louis, Mo.).

Human peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque fractionation of cell concentrates obtained from healthy donors seronegative for antibodies against HIV and hepatitis B virus; for individual experiments, the cells were stimulated with phytohemagglutinin (M form, 5 to 10 μg/ml) for 3 to 4 days in RPMI-10. Primary macrophages were prepared by countercurrent centrifugation elutriation of PBMCs and differentiation of the monocyte fraction (31); for individual experiments, cells were plated in flat-bottom 96-well microtiter plates as previously described (6).

Recombinant gene expression. Recombinant vaccinia viruses were derived from the Western Reserve (WR) wild-type strain; foreign genes were introduced into the thymidine kinase locus by standard methods (21). Transient gene expression was achieved either by infection with vaccinia virus recombinants containing the gene of interest or by transfection (with DOTAP [Boehringer Mannheim, Indianapolis, Ind.]) of infected cells with plasmids containing the gene linked to a suitable promoter. The multiplicity of infection was 10 PFU per cell for each vaccinia virus. To obtain negative controls not expressing the recombinant proteins of interest, cells were transfected with control plasmids or infected with WR. Infected cultures were incubated overnight at 31°C to allow accumulation of the recombinant proteins (37).

Viral envelope proteins and their receptors were produced by using previously described recombinant vaccinia viruses: for HIV-1 Envs, vCB-41 (LAV), vCB-43 (Ba-L), and vCB-16 (nonfusogenic uncleaved [Unc] Env) (4) (note that vCB-43 is a correction of the nomenclature used for Ba-L in reference 4); for CD4, vCB-3 (5); for CD46, vCB-48; for measles virus (MV) hemagglutinin (H) and fusion glycoprotein (F), vT7-H_{MV} and vT7-F_{MV}, respectively (38). In vCB-41, vCB-43, vCB-16, vCB-3, and vCB-48, the genes were linked to a synthetic strong early-strong late vaccinia virus promoter (10a); in vT7-H_{MV} and vT7-F_{MV}, they were linked to the T7 promoter.

For CD44S (standard or hemopoietic form), a plasmid containing the CDw44 cDNA (49) (donated by B. Seed, Harvard Medical School, Boston, Mass.) was digested with *Xho*I and *Xba*I. The excised fragment was digested with *Nco*I to remove 5′ untranslated sequences, filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and ligated into the *Stu*I site of plasmid pSC59 (10a). The resulting plasmid, designated pGA1-CD44S, contains the CD44S cDNA linked to the synthetic strong early-strong late vaccinia virus promoter. Recombinant CD44S was produced by transfection of pGA1-CD44S into cells that were also infected with vaccinia virus. For CD26 production, cells were infected with vaccinia virus recombinant vCB47-CD26; this virus was generated from plasmid pCB-47 (7), in which the cDNA is also linked to the synthetic strong early-strong late vaccinia virus promoter.

Bacteriophage T7 RNA polymerase was produced generally by infection with vTF7-3 (23) ($P_{7.5}$ natural early-late vaccinia virus promoter); alternatively, vP11gene1 (1) was used (P_{11} natural late vaccinia virus promoter). The *E. coli lacZ* gene linked to the T7 promoter was introduced into cells by infection with vaccinia virus recombinant vCB21R-LacZ. In this virus, the T7 promoter is followed by the 5′ untranslated region of encephalomyocarditis virus for improved expression (22) and the *lacZ* gene is followed by the T7 terminator. To produce vCB21R-LacZ, the region containing the T7 promoter, the encephalomyocarditis virus 5′ untranslated region, the multiple cloning site, and the T7 terminator was excised from plasmid pTM1 (36) and reinserted in the reverse orientation with respect to the flanking vaccinia virus thymidine kinase sequences (23a). The resulting plasmid was digested with *Eco*RI, filled in with the Klenow fragment of *E. coli* DNA polymerase I, and religated. The resulting plasmid was digested with *Bam*HI, and the 3-kb *Bam*HI fragment of the *E. coli lacZ* gene was inserted to produce plasmid pCB21R-LacZ, which in turn was used to generate vCB21R-LacZ. Background expression in the absence of T7 RNA polymerase was much lower when the expression cassette was introduced at the vaccinia virus thymidine kinase locus in the reverse orientation versus the forward orientation (data not shown). Where indicated, as an alternative to infection with vCB21R-LacZ, the *lacZ* gene was introduced by transfection with plasmid pG1NT7-β-gal (34a).

Analysis of protein production. Cell surface proteins were measured by using monoclonal antibodies (MAbs) and a FACScan flow cytometer (Becton Dickinson); results are expressed as mean fluorescence intensity. For CD4, direct staining was performed with fluorescein isothiocyanate-conjugated murine MAb OKT4A (Ortho Diagnostics, Raritan, N.J.). For other antigens, indirect staining with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim) was employed in conjunction with the following murine primary MAbs: for CD44, MAb A3D8 (Sigma); for CD26, MAb Ba5 (BioSource International, Camarillo, Calif.).

Recombinant CD44 in detergent cell lysates was analyzed by Western blotting (immunoblotting). Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. Proteins were transferred to nitrocellulose and stained with MAb A3D8 and then a horseradish peroxidase-conjugated secondary antibody (Boehringer Mannheim). Specific bands were visualized by chemiluminescence detection.

Cell fusion assay. Fusion was analyzed by a previously described reporter gene activation assay (37) in which the cytoplasm of the CD4-expressing cell population contained T7 RNA polymerase (encoded by vTF7-3) and the cytoplasm of the Env-expressing population contained the *E. coli lacZ* gene linked to the T7 promoter (in vCB21R-LacZ); β-galactosidase (β-Gal) was produced selectively in fused cells. Cytosine arabinoside (40 μg/ml) was added to the fusion reaction mixture to reduce nonspecific β-Gal production. The designated cell populations were mixed in individual wells of 96-well microtiter plates. Typically, the ratio of CD4-expressing to Env-expressing cells was 1:1 (2×10^5 total cells per well, 0.2-ml total volume); with PBMCs as the CD4-expressing cell type, the ratio was 5:1. Cultures were incubated for 2.5 to 3 h to allow fusion; β-Gal was assessed by quantitative colorimetric assay of detergent cell lysates or by in situ staining. Where indicated, CD4-expressing cells were incubated with the designated MAbs for 1 h at room temperature prior to being mixed with Env-expressing cells.

Fusion with transient cell hybrids. Fusion experiments with transient cell hybrids involved two steps. First, transient hybrids were formed by mixing the two designated cell types, one expressing vaccinia virus-encoded CD4; in addition, one cell type expressed vaccinia virus-encoded MV H and F (plus T7 RNA polymerase) and the other expressed vaccinia virus-encoded CD46. The cultures were incubated for 1 h at 37°C to allow hybrid formation. The advantage of the MV-CD46 protocol is that hybrids are formed only heterotypically between the desired cell types and not homotypically with the same cell type. In the second step, another cell population expressing vaccinia virus-encoded Envs (and containing the *lacZ* gene linked to the T7 promoter) was added. Following incubation for 2.5 to 3 h at 37°C, fusion was scored by the sensitive method of in situ staining for β-Gal. Details of the experiments are provided in the figure legends.

RESULTS

Our experimental approach involved analysis of fusion between two distinct cell populations, one expressing CD4 (endogenous or vaccinia virus encoded) and the other expressing the indicated HIV-1 Env (vaccinia virus encoded). Fusion was scored by a reporter gene activation assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other contained the *lacZ* gene linked to the T7 promoter; cell fusion activates β-Gal production (37). The Envs from LAV (Lai) and Ba-L served as prototypes for T-cell line-tropic and macrophage-tropic isolates, respectively; the nonfusogenic Unc Env was used as a negative control. Our previous findings indicated that with CD4-expressing continuous human cell lines (e.g., T-cell lines and CD4-expressing HeLa cells) as targets, cell fusion was much more efficient with the LAV Env than with the Ba-L Env; with primary macrophage targets, the opposite selectivity was observed (4).

Fusion with human promyelocytic and monocytic cell lines. As one experimental approach, we examined fusion with human $CD4^+$ continuous cell lines that are conditionally infectible with macrophage-tropic HIV-1 isolates. Untreated HL-60 promyelocytic cells are reportedly infectible with T-cell line-tropic but not macrophage-tropic HIV-1 isolates; when treated with agents that promote differentiation along the myelocytic or monocytic pathway, the cells acquire susceptibility to macrophage-tropic isolates while retaining susceptibility to T-cell line-tropic strains (30). Retinoic acid is particularly effective at promoting this change. These findings prompted us to examine the effects of differentiating agents on the ability of these cells to undergo fusion mediated by recombinant Envs from T-cell line-tropic versus macrophage-tropic HIV-1 iso-

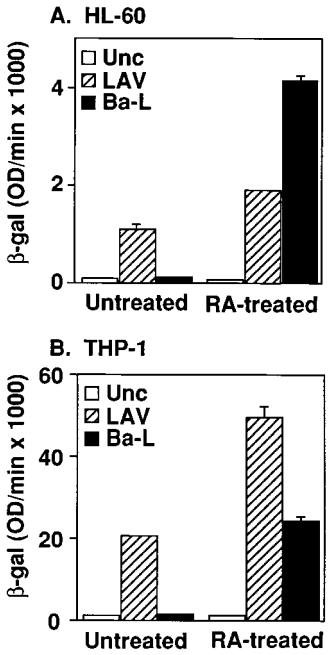

FIG. 1. Effect of retinoic acid (RA) treatment on cell fusion with promyelocytic and monocytic cell lines. Untreated or retinoic acid-treated HL-60 cells (A) or THP-1 cells (B) expressed vaccinia virus-encoded CD4 and T7 RNA polymerase. A separate population of cells (HeLa) expressed the designated vaccinia virus-encoded Envs and contained the *lacZ* gene linked to the T7 promoter. Cell mixtures (duplicates) were incubated at 37°C for 2.5 h. Fusion was assessed by measurement of β-Gal in detergent cell lysates. The values within each panel were obtained from separate samples in the same experiment. Error bars indicate the standard deviations of the mean values obtained from duplicate fusion assays. OD, optical density.

lates. Figure 1 shows results obtained by using the quantitative assay of β-Gal in detergent cell lysates. With untreated HL-60 cells (Fig. 1A), fusion was readily observed with the LAV Env, whereas only background β-Gal activity was observed with the Ba-L Env (similar to the Unc Env negative control). Cells pretreated for 5 days with retinoic acid retained permissiveness for fusion with the LAV Env and, most notably, acquired strong permissiveness for fusion with the Ba-L Env. In repeated experiments, fusion mediated by the LAV Env showed variable effects of retinoic acid treatment; typically, fusion was stimulated to a degree similar to or greater than that shown in Fig. 1A but in some cases it was inhibited. The most consistent findings were that untreated cells were permissive for LAV but not Ba-L Env-mediated fusion and that retinoic acid-treated cells acquired strong permissiveness for Ba-L. We also observed that HL-60 cells acquired Ba-L Env fusion permissiveness when treated with phorbol 12-myristate 13-acetate, another differentiating agent (data not shown). Our results parallel the report that differentiating agents render HL-60 cells susceptible to infection by macrophage-tropic isolates (30). We also conducted experiments with the human monocytic cell line THP-1. Like HL-60, these cells are reportedly susceptible to infection by T-cell line HIV-1 isolates (30, 33) and become susceptible to macrophage-tropic isolates only after treatment with differentiating agents (33). Consistent with this, we observed that untreated THP-1 cells supported fusion by the LAV but not the Ba-L Env; retinoic acid treatment rendered these cells permissive for Ba-L Env-mediated fusion (Fig. 1B). The results reported in this section demonstrate that treatment of promyelocytic and monocytic cell lines with differentiating agents induces changes in fusion permissiveness that parallel the reported changes in susceptibility to HIV-1 infection. They also indicate differential regulation of fusogenic specificity for T-cell line-tropic versus macrophage-tropic strains.

Fusion specificities of transient cell hybrids. The results presented above, coupled with our previous findings that T-cell line versus macrophage tropism is associated with the fusogenic specificity of Env (4), prompted us to test directly the model in which tropism results from distinct fusion cofactors differentially expressed in various human target cell types. We conducted experiments with transient cell hybrids formed between a CD4-expressing nonhuman (fusion-nonpermissive) cell type and various human cell types that are selectively permissive for fusion with different classes of Envs (Fig. 2A). Suspensions of murine NIH 3T3 cells expressing vaccinia virus-encoded T7 RNA polymerase (plus or minus vaccinia virus-encoded CD4) were prepared; to enable hybrid formation, these cells also expressed vaccinia virus-encoded MV F and H. The partner cells for hybrid formation were monolayers of either HeLa cells or primary human macrophages expressing vaccinia virus-encoded CD46 (the primary MV receptor). The NIH 3T3 cell suspensions were added to the monolayer cells in various combinations. After incubation for 1 h at 37°C to allow transient hybrid formation, cells expressing vaccinia virus-encoded Envs and containing the *lacZ* gene linked to the T7 promoter were added. The cultures were incubated for 2.5 h at 37°C; fusion was assessed by the sensitive in situ assay for β-Gal. The results in Fig. 2A reveal that the permissiveness of the transient hybrids for fusion mediated by the different Envs depended on the partner cell type used to form the hybrids. When hybrids were prepared by using HeLa cells, fusion occurred with the LAV Env (panel a) but not with the Ba-L Env (panel b) (or with the negative control Unc Env [panel c]). When hybrids were prepared by using primary macrophages, the opposite selectivity was observed: fusion occurred with the Ba-L Env (panel f) but not with the LAV Env (panel e). Fusion was CD4 mediated, as judged by the results obtained when vaccinia virus-encoded CD4 was omitted from the NIH 3T3 cells (panels d and h). Additional controls verified that fusion was strictly dependent on transient hybrid formation, since β-Gal was not produced when the NIH 3T3 cells expressed MV H in the absence of F (data not shown for this experiment, but see Fig. 2B). The simplest interpretation of these findings is that each fusion partner provided a distinct cofactor(s) that enabled the CD4 expressed on the NIH 3T3 cell to function in the fusion reaction with the appropriate Env. This conclusion is complicated somewhat with primary macrophages as hybrid partners, since these cells have a low level of endogenous CD4 that is sufficient to support fusion by Envs from macrophage-tropic isolates (4). Thus, it could be argued that the β-Gal fusion signal was due not to the transfer of a cofactor to the CD4-expressing NIH 3T3 cells but instead to the transfer to the T7 RNA polymerase to the macrophages. Indeed, we observed a low level of fusion with the Ba-L Env in the absence of CD4 on NIH 3T3 cells (panel h). However, fusion was considerably greater when recombinant CD4 was expressed on NIH 3T3 cells (compare panels f and h). Quantitative analysis of detergent cell lysates indicated greater-than-fourfold higher levels of β-Gal when NIH 3T3 cells expressed CD4 (data not shown). This finding suggests that the macrophages indeed provided a cofactor that enabled the CD4 on NIH 3T3 cells to support fusion with the Ba-L Env.

We extended this type of analysis to transient hybrids formed between two human cell types with distinct permissiveness for different types of HIV-1 isolates. In the experiment

FIG. 2. Fusion specificities of transient cell hybrids. In 96-well flat-bottom microtiter plates, the designated cell types were used to generate transient hybrids expressing vaccinia virus-encoded CD4 and T7 RNA polymerase. Another cell population (NIH 3T3) expressing the indicated vaccinia virus-encoded Envs and containing the *lacZ* gene were added to the hybrids in the presence of cytosine arabinoside. The cells were mixed by pipetting, incubated, and stained in situ for β-Gal. Transient hybrid formation and fusion reactions were conducted as follows. (A) NIH 3T3 cell suspensions expressing vaccinia virus-encoded T7 RNA polymerase (vP11gene1), MV H and F, and CD4 (panels a to c and e to g) were prepared; control cells did not express CD4 (panels d and h). The designated cell suspensions (3×10^4 cells per well) were added to monolayers ($\sim 10^5$ cells per well) of HeLa cells (panels a to d) or macrophages (panels e to h) expressing vaccinia virus-encoded CD46. Following 1 h of incubation at 37°C to allow transient hybrid formation, cells expressing the indicated vaccinia virus-encoded Envs and containing the *lacZ* gene (plasmid pG1NT7-β-Gal) were added (10^5 cells per well). Staining was performed after 2.5 h of incubation at 37°C. (B) HeLa cell suspensions expressing vaccinia virus-encoded T7 RNA polymerase (vTF7-3) and either MV H and F (left panels) or H alone (right panels) were prepared. The HeLa cells were added (4×10^4 per well) to macrophage monolayers ($\sim 10^5$ cells per well) expressing vaccinia virus-encoded CD46 plus CD4. Following incubation for 1 h at 37°C to allow transient hybrid formation, cells (NIH 3T3) expressing the indicated vaccinia virus-encoded Envs and containing the *lacZ* gene (vCB21R-LacZ) were added (10^5 per well). Staining was performed after 3 h of incubation at 37°C.

whose results are shown in Fig. 2B, suspensions of HeLa cells expressing vaccinia virus-encoded T7 RNA polymerase (and MV F plus H, left panels; H alone, right panels) were added to macrophage monolayers expressing vaccinia virus-encoded CD4 (and CD46). After incubation for 1 h to allow hybrid formation, cells expressing the Envs indicated in Fig. 2B (and containing the *lacZ* gene) were added. The cultures were incubated for 3 h at 37°C and then stained for β-Gal. Under conditions enabling hybrid formation (H plus F [left panels]), fusion occurred with both the LAV and Ba-L Envs (but not with the control Unc Env); by contrast, no fusion occurred under conditions in which hybrid formation was not promoted (H alone [right panels]). Taken together, the findings in this section indicate a close correspondence between the fusion selectivity of a transient cell hybrid and the cell type used for its formation.

Evidence against involvement of CD44 or CD26 in fusion mediated by Envs from macrophage-tropic HIV-1 isolates. In view of our functional results suggesting the existence of a distinct fusion cofactor(s) for Envs from macrophage-tropic HIV-1 isolates, it was important to directly test the possible roles of specific surface glycoproteins recently proposed to be involved in the entry of these strains. The adhesion molecule CD44 (lymphocyte homing receptor) was implicated initially on the basis of the ability of anti-CD44 MAbs to block infection by macrophage-tropic (including Ba-L) but not T-cell line-tropic (Lai) isolates (45). A subsequent study (20) reported that CD44-negative Jurkat T cells, normally infectible only by T-cell line-tropic isolates, become susceptible to macrophage-tropic isolates (including Ba-L) upon transformation with cDNA encoding the standard (hemopoietic) isoform of CD44 (CD44S); this effect was not observed with the epithelial isoform (CD44E). We applied similar criteria to test for a direct

TABLE 1. Effect of anti-CD44 and anti-CD4 MAbs on Env-mediated cell fusion

MAb	MAb concn (μg/ml)	β-Gal activity (% of control) in RA-treated HL-60 cells[a]		MAb concn (μg/ml)	β-Gal activity (% of control) in PBMCs[a]	
		LAV[b]	Ba-L[b]		LAV[b]	Ba-L[b]
None	0	100[c,d]	100[c,e]	0	100[c,f]	100[c,g]
Anti-CD44	10	104	94	5	117	116
Anti-CD4	2.5	3	3	2.5	0	0.5

[a] HL-60 cells were coinfected with vTF7-3 and vCB3. PBMCs were infected with vTF7-3.
[b] Envs were expressed on HeLa cells which also contained the *lacZ* gene (vCB21R-lacZ).
[c] Defined as 100% of the β-Gal level obtained in the absence of an antibody.
[d] The actual level was 27.3×10^3 U of optical density per min.
[e] Actual level: 7.8×10^3 U of optical density per min.
[f] Actual level: 190×10^3 U of optical density per min.
[g] Actual level: 28×10^3 U of optical density per min.

TABLE 2. Flow cytometry analysis of cell surface recombinant CD44 produced by using the vaccinia virus system[a]

MAb	Jurkat cells[b]		NIH 3T3 cells[b]	
	Control	CD44S	Control	CD44S
Isotype control	4	4	14	12
Anti-CD44	6	105	18	508

[a] The values shown are mean fluorescence intensity in arbitrary units.
[b] Cells were infected with vCB-3 and transfected with either control plasmid pSC59 or plasmid pGA1-CD44S.

TABLE 3. Flow cytometry analysis of endogenous CD44 and CD4[a]

MAb	HeLa cells	HL-60 cells	
		Untreated	Retinoic acid treated
Isotype control	5	12	12
Anti-CD44	867	430	400
Isotype control	ND[b]	4	4
Anti-CD4	ND	64	64

[a] The values shown are mean fluorescence intensity in arbitrary units.
[b] ND, not done.

role of CD44 in fusion mediated by Envs from macrophage-tropic isolates. Table 1 shows that high concentrations of MAb A3D8 (reported to block infection by macrophage-tropic isolates [45]) had no inhibitory effects on fusion mediated by the Ba-L Env (or the LAV Env) when tested against either PBMCs or retinoic acid-treated HL-60 cells. By contrast, anti-CD4 MAb OKT4A showed the expected potent inhibition of fusion mediated by either Env. As an independent approach, we examined the effects of recombinant CD44S expression on fusion mediated by the Ba-L Env. Jurkat cells (the same cell type used in the study described in reference 20) or NIH 3T3 cells were transfected with either control plasmid pSC59 or plasmid pGA1-CD44S containing the CD44S cDNA linked to a vaccinia virus promoter; the cells were also infected with a vaccinia virus recombinant encoding CD4. Flow cytometry (Table 2) verified that for each cell type, the vaccinia virus system yielded good CD44 surface expression. Western blot analysis of detergent cell lysates (Fig. 3) confirmed that the recombinant protein had the expected mobility for CD44S of ~80 kDa (50), identical to that of the endogenous CD44S in HeLa cells. When fusion was examined (Fig. 4A), control Jurkat cells showed the expected pattern of permissiveness for the LAV but not the Ba-L Env. Recombinant CD44 had no influence on the fusion pattern; LAV fusion occurred normally, and Ba-L fusion was still negative. CD44 was similarly ineffective when expressed on NIH 3T3 cells; fusion did not occur with either Env for control cells or CD44-expressing cells (data not shown). Finally, we point out that endogenous CD44 levels did not correlate with permissiveness for Ba-L fusion. Thus, HeLa cells, which are nonpermissive for Ba-L fusion, expressed high levels of endogenous CD44 at the cell surface (Table 3); Western blot analysis (Fig. 3) confirmed that it was the ~80-kDa CD44S isoform and not the ~130-kDa CD44E isoform (50) in these cells. With HL-60 cells, there was no change in endogenous surface CD44 levels upon retinoic acid treatment (Table 2), despite the acquisition of Ba-L fusion permissiveness. Also, surface CD4 levels were unaffected by retinoic acid (Table 3), in agreement with previously published results (30).

Another molecule of interest is the CD26 antigen (dipeptidyl peptidase IV). CD26 has received an extraordinary amount of attention because of an initial report proposing a role in HIV entry (8); however, subsequent reports by numerous laboratories failed to corroborate this notion (2, 7, 9, 41, and numerous later reports). These studies were performed with T-cell line-tropic isolates and their corresponding Envs. A recent report (40) suggested a role for CD26 in infection by macrophage-tropic but not T-cell line-tropic isolates on the basis of studies with the PM-1 cell line, which supports replication by both types of isolates. Infection of these cells with macrophage-tropic (including Ba-L) but not T-cell line-tropic isolates led to preferential depletion of cells expressing high levels of CD26. Moreover, when PM-1 cells were sorted and cloned on the basis of CD26 expression, the replication efficiency of a macrophage-tropic isolate was found to correlate positively with CD26 levels. These findings prompted us to test the effects of recombinant CD26 expression on fusion mediated by an Env from a macrophage-tropic isolate. For these experiments, we examined both HL-60 and NIH 3T3 cells, types shown above to be rendered permissive for Ba-L Env-mediated fusion by the appropriate treatment (retinoic acid stimulation for HL-60 and transient hybrid formation for NIH 3T3). These cells were coinfected with one vaccinia virus recombinant encoding CD4 and another encoding CD26 (or with strain WR as a negative control). Flow cytometry analysis confirmed CD26 surface expression (mean fluorescence intensity of 1,310 for the CD26 virus compared with 114 for strain WR), consistent with our previous findings obtained by using plasmid transfection (7). The results shown in Fig. 4B indicate that CD26 expression did not confer Ba-L Env fusion permissiveness on untreated HL-60 cells; similarly, no fusion stimulation was observed with retinoic acid-treated HL-60 cells or with NIH 3T3 cells (data not shown). We conclude that expression of recombinant CD26 was insufficient to confer Ba-L Env fusion permissiveness on cell types that could be rendered permissive by other treatments.

DISCUSSION

We used a recombinant vaccinia virus expression and assay system to test the model in which the distinct fusion specificities of Envs from T-cell line-tropic versus macrophage-tropic isolates of HIV-1 are due to the presence of different fusion cofactors on various $CD4^+$ target cells. Two supportive lines of evidence were obtained. The first was based on studies of promyelocytic and monocytic cell lines, for which it is possible to experimentally modulate permissiveness for infection by different HIV-1 isolates. We observed a close parallel between previously reported differentiation-induced changes in infection susceptibility for T-cell line-tropic versus macrophage-tropic HIV-1 isolates and changes in permissiveness for fusion mediated by the corresponding Envs. These results suggest that major determinants of infection susceptibility are at the level of the membrane fusion reactions associated with virion entry and cell fusion. Indeed, this conclusion is in accord with virus infection studies suggesting that the block under nonpermissive conditions is at an early step, perhaps entry (33). The fusion restrictiveness of untreated HL-60 cells for the LAV Env and the acquisition of Ba-L fusion permissiveness upon retinoic acid treatment are consistent with the notion of distinct fusion cofactors. It is important to point out that in these cell lines, the differentiation-associated changes in infection-fusion susceptibility are not due to modulation of CD4 expression. Flow cytometry analysis results reported by others (30) and confirmed by us indicated that retinoic acid treatment of HL-60 cells is not accompanied by alterations in surface CD4

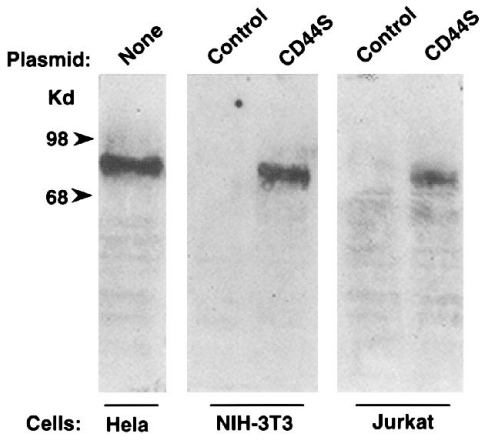

FIG. 3. Western blot analysis of CD44. HeLa cells were tested directly; Jurkat and NIH 3T3 cells were transfected with either control plasmid pSC59 or pGA1-CD44S. Detergent cell lysates were subjected to Western blot analysis with anti-CD44 MAb A3D8. The numbers at the left indicate the positions of protein standards. Kd, kilodaltons.

levels. Moreover, in this study the differentiation-induced changes in fusion permissiveness for the LAV versus the Ba-L Env were observed under conditions of vaccinia virus-mediated elevation of CD4 levels on both untreated and treated cells. Other determinants must, therefore, be responsible.

A second, more direct line of evidence emerged from experiments with transient cell hybrids. With hybrids formed between a CD4-expressing nonhuman (nonpermissive) cell type (murine NIH 3T3 cells) and various differentially permissive human cell types (HeLa cells or macrophages), we observed a close parallel between the fusion specificity of a hybrid and the human cell type used for its formation. Furthermore, hybrids formed between two human cell types that differ in fusion permissiveness (HeLa cells and macrophages) had the composite permissiveness of both partners. These results argue that the fusion blocks with discordant target cell-Env combinations are not due to the presence of fusion inhibitors in the nonpermissive cells. Instead, they provide direct support for the simple model in which fusion requires cofactors that are distinct for Envs from T-cell line-tropic versus macrophage-tropic HIV-1 isolates; infection cytotropism results from the differential expression of these cofactors in various $CD4^+$ target cell types. This model is easily reconciled with the fact that T-cell line versus macrophage tropism is not absolute; most HIV-1 strains, including primary isolates obtained directly from infected individuals, have some dual tropic character, preferentially infecting one of these target cell types but having some capacity to infect the other (14, 52). Conversely, some target cells (e.g., primary $CD4^+$ T cells) are comparably permissive for both classes of isolates. These departures from a simple all-or-none model are readily accommodated by considering that a given Env may strongly favor one cofactor yet display some activity with the other. Furthermore, some target cell types (e.g., T-cell lines and primary macrophages) may express one cofactor preferentially over the other but not exclusively; other cell types (e.g., primary $CD4^+$ T cells) may express sufficient levels of both. We emphasize that our results do not distinguish between single and multiple fusion cofactors capable of functioning for each class of Envs.

We applied the fusion assay system to assess directly the role of two specific molecules previously implicated in infection by macrophage-tropic HIV-1 isolates. Our findings do not support a role for CD44, since the A3D8 MAb reported by others to block infection of macrophages (45) had no effect on fusion. Moreover, we found that expression of recombinant CD44S failed to confer Ba-L permissiveness to Jurkat cells, in contrast to a previous study based on infection susceptibility of Jurkat-CD44 transformant cells (20). Similarly, with NIH 3T3 cells, no effects of recombinant CD44S were observed in either our study or the earlier study (20). There are other inconsistencies with the CD44 notion. The reported inhibition of macrophage-tropic isolate infection by anti-CD44 MAbs was observed with primary macrophages but not other susceptible target cells (20, 45); most notably, the effects of the antibodies on T-cell line-tropic versus macrophage-tropic isolates were not compared in a single target cell type that is susceptible to both types of strains. Furthermore, there is little correlation between endogenous CD44 levels and susceptibility to macrophage-tropic isolates. For example, as we and others (20) have noted, HeLa cells express abundant CD44 levels yet expression of recombinant CD4 renders these cells permissive for fusion with T-cell line-tropic but not macrophage-tropic Envs. Similarly, retinoic acid induces susceptibility to macrophage-tropic isolates without modulating endogenous CD44 levels. It is difficult to reconcile these findings with a direct role for CD44 in the fusion process, although indirect effects at other stages of the infection cycle remain possible (20). Regarding CD26, we found that expression of the recombinant protein (along with CD4) failed to confer Ba-L Env fusion permissiveness on either HL-60 or NIH 3T3 cells, types that can be rendered permissive

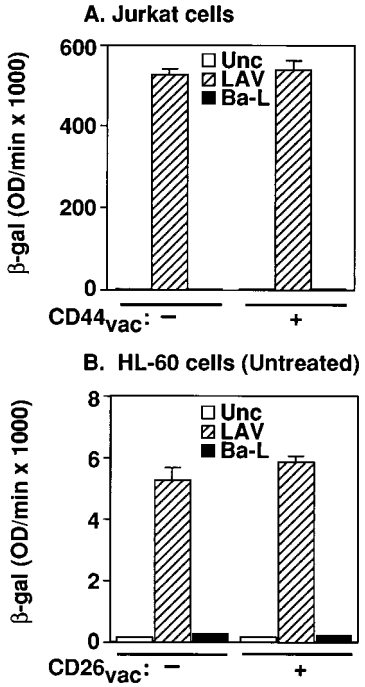

FIG. 4. Lack of effect of recombinant CD44 or CD26 on fusion. (A) Jurkat cells were transfected with either control plasmid pSC59 (−) or pGA1-CD44S (+) and infected with vaccinia virus recombinants encoding CD4 and T7 RNA polymerase. A separate population of cells (HeLa) expressed the designated vaccinia virus-encoded Envs and contained the *lacZ* gene linked to the T7 promoter. Cell mixtures (duplicates) were incubated at 37°C for 2.5 h. Fusion was assessed by measurement of β-Gal in detergent cell lysates. (B) Untreated HL-60 cells were infected with either control vaccinia virus strain WR (−) or vCB47-CD26 (+). Incubation with Env-expressing cells and assay of β-Gal were performed as for panel A. The values within each panel were obtained from separate samples in the same experiment. Error bars indicate the standard deviations of the mean values obtained from duplicate fusion assays. OD, optical density.

by other treatments (retinoic acid and transient hybrid formation, respectively). Moreover, several $CD4^+$ human cell types that are positive for CD26 surface expression support infection-fusion by T-cell line-tropic but not macrophage-tropic isolates. Thus, the reported correlation between CD26 expression and susceptibility to infection by macrophage-tropic isolates in a particular cell line (40) probably reflects an indirect role for CD26 or fortuitous coregulation of CD26 and the factor of interest.

Our findings supporting the model in which HIV-1 tropism for T-cell lines versus macrophages is due to distinct cell type-specific fusion cofactors highlights the critical importance of identifying the molecules in question. It should be noted that the cofactor for T-cell line-tropic isolates has been reported to be protease resistant; this suggests the possibility that the cofactor is not a protein, although other interpretations have been offered (19). We are developing functional cDNA cloning strategies by using vaccinia virus-based expression and assay systems in the hope of detecting and isolating cDNAs responsible for the fusion specificities underlying HIV-1 tropism for T-cell lines versus primary macrophages.

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ADDENDUM IN PROOF

We have directly comfirmed the model for HIV-1 cytotropism proposed herein by demonstrating that selective fusion cofactor activity is associated with two distinct G protein-coupled receptors: fusin, which functions preferentially for T-cell line-tropic isolates (Y. Feng, C. C. Broder, P. E. Kennedy, and E. A. Berger, Science **272:**872–877, 1996), and the chemokine receptor CC CKR5, which functions preferentially for macrophage-tropic isolates (G. Alkhatib, C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger, Science **272:**1955–1958, 1996).

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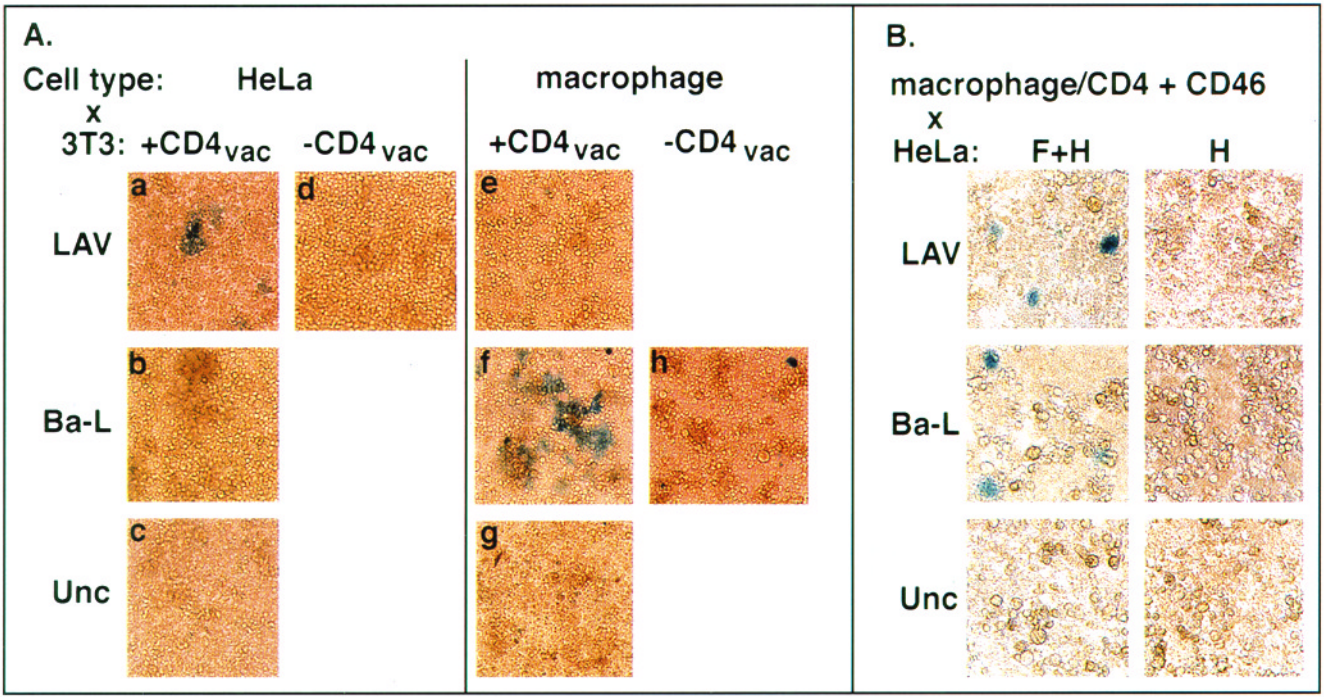
A.
Cell type: HeLa
x
3T3: +CD4 vac
-CD4 vac
macrophage
+CD4 vac
-CD4 vac
LAV
Ba-L
Unc
a
b
c
d
e
f
g
h
B.
macrophage/CD4 + CD46
x
HeLa: F+H
H
LAV
Ba-L
Unc