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A Seven-Transmembrane Domain Receptor Involved in Fusion and Entry of T-Cell-Tropic Human Immunodeficiency Virus Type 1 Strains

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Entry of human immunodeficiency virus type 1 (HIV-1) into cells requires binding to CD4 and fusion with a cellular membrane. Fusion does not occur in most nonhuman cells even when they express human CD4, indicating that one or more human accessory factors are required for virus infection. Recently, a seven-transmembrane domain protein has been shown to serve as an accessory factor for T-cell-tropic (T-tropic) HIV-1 isolates (Y. Feng, C. C. Broder, P. E. Kennedy, and E. A. Berger, Science 272:872–877, 1996). Here we show that expression of this glycoprotein, termed fusin, in murine, feline, simian, and quail cell lines, in conjunction with human CD4, rendered these cells fully permissive for HIV-1 envelope glycoprotein (Env)-mediated membrane fusion. Expression of CD4 or fusin alone did not permit fusion. In addition, introduction of fusin and CD4 into a human cell line, U87MG, that is resistant to HIV-1-induced syncytium formation and to infection by HIV-1 when expressing CD4 alone made this cell line permissive for Env-mediated cell-cell fusion. Fusion was observed only with T-tropic Env proteins. Macrophage-tropic (M-tropic) Env proteins from the SF162, ADA, and Ba-L HIV-1 strains did not fuse with cells expressing fusin and CD4, suggesting that M-tropic viruses utilize an accessory molecule other than fusin. Finally, coexpression of fusin and CD4 made both a murine and feline cell line susceptible to virus infection by T-tropic, but not M-tropic, HIV-1 strains.

The envelope protein (Env) of human immunodeficiency virus type 1 (HIV-1) binds virus to the cell surface via a high-affinity interaction with CD4. A subsequent conformational change results in fusion between the viral envelope and a cellular membrane (for a review, see reference 44). While Env-CD4 interactions have been well characterized, it is clear that binding to CD4 by itself is not sufficient for the subsequent membrane fusion reaction (40). Expression of human CD4 (huCD4) in nonhuman cells generally does not render them susceptible to either virus infection or Env protein-mediated syncytium formation (2, 5, 13, 16, 17, 40). Species restriction to infection is often at the level of virus entry and is unidirectional in nature; human cells bearing CD4 readily form syncytia with nonhuman cells bearing Env (2, 13). Furthermore, there are several examples in which expression of CD4 in human cell lines fails to render them permissive for HIV-1 entry and Env-mediated membrane fusion (12, 13, 30). Finally, HIV-1 strains can sometimes be classified as T-cell-tropic (T-tropic) or macrophage-tropic (M-tropic), depending on their differential abilities to infect these CD4-positive target cells (11, 19, 60). Thus, expression of CD4 does not necessarily lead to membrane fusion.

Cells that are nonpermissive for Env-mediated membrane fusion can be made permissive in several ways. Transient heterokaryons formed between murine cell lines expressing huCD4 and CD4-negative human cells support both HIV-1 entry and syncytium formation (5, 16). This finding indicates that one or more components (or accessory factors) in human cells can, when delivered to nonhuman cells, render them susceptible to HIV-1 infection. That the accessory factor(s) is a component of the plasma membrane was shown by Dragic et al. (17) who found that fusion of human erythrocyte ghosts with nonpermissive cells rendered them susceptible to HIV-1 infection and membrane fusion. While identification of the accessory factor(s) has elicited considerable interest, none of the molecules proposed to serve this role have proven to be required for HIV-1 infection, and some, like CD26, appear to have no role at all in HIV-1 entry (1, 6, 8, 21, 38, 52, 62, 63).

A seven-transmembrane domain protein has recently been reported to serve as an accessory factor for T-tropic, but not M-tropic, HIV-1 strains (23). It has been proposed that the protein, which has been referred to as 7TMS (22), LESTR (39), L5 (32), HM89 (50), and HUMSTR (48), be termed fusin as a consequence of its ability to assist HIV-1 Env-mediated membrane fusion (23). Fusin is widely distributed in human tissues and is expressed at high levels in human peripheral blood mononuclear cells and in a number of hematopoietic cell lines including Jurkat and HL-60 cells (22, 32, 35, 39, 48, 50). Fusin bears approximately 33% homology with members of the CXC and CC chemokine receptor families, though studies with a large number of chemokines have failed to reveal functional interactions with fusin (32, 35, 39, 50). To confirm and extend the findings of Feng et al. (23), we examined the ability of fusin to support Env-mediated syncytium formation and HIV-1 infection in a variety of cell types. Here we show that expression of fusin in a number of nonhuman cell lines, in conjunction with huCD4, renders them fully permissive to T-tropic, but not M-tropic, HIV-1 Env-induced syncytium formation and virus infection. Identification of this accessory factor has important

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implications for understanding viral tropism and the mechanisms underlying Env-induced membrane fusion.

MATERIALS AND METHODS

Constructs. Fusin cDNA was cloned from a human fetal spleen cDNA library as previously described (22). In order to facilitate cloning, fusin was subcloned into pSP73 (Promega) by using the upstream *Eco*RI site and the downstream *Kpn*I site provided by the original fusin cloning vector. Fusin was subcloned into the vaccinia virus expression vector pSC59 by using the upstream *Eco*RI site and the downstream *Xba*I site provided by pSP73. Ligation into pSC59 employed *Eco*RI and *Spe*I sites, resulting in the elimination of the *Xba*I and *Spe*I sites. pSC59 provides protein expression driven by the vaccinia virus synthetic early/late promoter as previously described (18). Fusin was also excised with *Eco*RV and *Xho*I and subcloned into the mammalian expression vector pREP8 (Invitrogen) that had been digested with *Hin*dIII, blunted with a Klenow fill-in reaction in order to accommodate the *Eco*RV site, and cut with *Xho*I.

The influenza virus hemagglutinin (HA) affinity tag was introduced at the C terminus of fusin by first creating a unique *Hin*dIII restriction site at the 3′ end of the open reading frame, followed by oligonucleotide annealing. PCR primer LESTR2 (GGCCAAGGAAGCTGTTGGCTG) was positioned upstream of the unique *Bam*HI site within fusin, while LESTR1 (AGCTCCCGGGAAGCTTG AGTGAAAACTTGAAGACTCA) was designed to introduce a unique *Hin*dIII restriction site just before the stop codon of fusin while preserving the original amino acid sequence. PCR was performed with primers LESTR1 and LESTR2, and the resulting product was digested with the restriction enzymes *Bam*HI and *Hin*dIII and cloned into the corresponding position in pSP73/Fusin. The resulting plasmid, pSP73/Fusin-Hind, was digested with *Hin*dIII, and the oligonucleotides LESTR5 (AGCTACGATGTTCCGGATTACGCATCTCTTCCCGGG) and LESTR6 (AGCTCCCGGGAAGAGATGCGTAATCCGGAACATCGT) were ligated to introduce the HA tag (YDVPDYASL), which was followed by a stop codon, eliminating the *Hin*dIII site and introducing a new *Xma*I site downstream of the stop codon. The resulting tagged form of fusin (fusin-HA) was subcloned into the vaccinia virus expression vector pSC65 by using the upstream *Bgl*II site provided in pSP73 and the downstream *Xma*I site introduced by the oligonucleotides (18).

Cells. The human cervical carcinoma cell lines HeLa and HeLaT4$^+$ (40) and the human astroglioma cell line U87MG were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID). The feline kidney cell lines CCCS+L− and CCCS+L−CD4 (42) and the murine embryo fibroblast cell line PA317T4 (42) were a kind gift from James Simon, University of Pennsylvania. The Japanese quail fibrosarcoma cell line QT6 (ATCC CRL-1708) was provided by John Balliet, University of Pennsylvania. The following cell lines were obtained from the American Type Culture Collection: NIH 3T3, murine embryo cells (CRL-1658); B-SC-1, African green monkey kidney cells (CCL-26); CV-1, African green monkey kidney cells (CCL-70), and human HuTK− 143B fibroblasts (CRL-8303).

Tissue culture media and supplements were purchased from Life Technologies, Inc., unless otherwise noted. HeLa, HeLaT4$^+$, CCCS+L−, CCCS+L−CD4, PA317T4, NIH 3T3, and HuTK− cell lines were maintained in Dulbecco's modified Eagle medium with a high level of glucose and without L-glutamine, supplemented with 10% fetal bovine serum (Hyclone). CD4 expression was selected for by the addition of 0.5 mg of G418 per ml to the media of the HeLaT4$^+$ and PA317T4 cells and by the addition of 0.2 mg of hygromycin (Boehringer Mannheim Biochemicals) per ml to the medium of the CCCS+L−CD4 cells. B-SC-1, CV-1, and U87MG cells were maintained in minimum essential medium supplemented with 10% iron-enriched bovine calf serum (Intergen). QT6 cells were maintained in medium 199, supplemented with 10% tryptose phosphate broth (Sigma), 5% fetal bovine serum, and 1% chicken serum. All media were supplemented with 2 mM glutamine and penicillin-streptomycin.

Viruses. Christopher Broder of the NIAID kindly provided us with the following panel of recombinant vaccinia viruses encoding the Env proteins of various T- and M-tropic HIV-1 strains (indicated in parentheses) (4): T-tropic recombinants, vSC60 (IIIB), vCB34 (SF2), vCB36 (RF), and vCB41 (Lai, LAV); M-tropic recombinants, vCB32 (SF162), vCB39 (ADA), and vCB43 (Ba-L). The recombinant vaccinia viruses vCB16 (encoding a nonfusogenic, uncleaved IIIB Env) and vSC8 (encoding LacZ) were used as negative controls. Christopher Broder also provided us with the recombinant viruses vCB3 (4) (encoding huCD4), vTF1.1 (encoding the T7 polymerase), and vCB21r (encoding *lacZ* under control of the T7 promoter). The recombinant vaccinia virus vBD4, expressing fusin-HA under control of the vaccinia virus synthetic early/late promoter, was generated by standard techniques utilizing thymidine kinase-negative selection (18). The HIV-1 strains used in this study were the T-tropic virus HxB2 (a kind gift from F. Gonzalez-Scarano, University of Pennsylvania) and the M-tropic virus Ba-L received through the AIDS Research and Reference Reagent Program (25). Virus stocks of HxB2 were prepared in H9 cells cultured in RPMI 1640 with 10% fetal bovine serum. The culture supernatant was collected 7 to 10 days postinfection and filtered through a 0.45-μm-pore-size filter to remove cell debris. Ba-L was amplified in phytohemagglutinin-stimulated peripheral blood mononuclear cells from healthy human volunteers and prepared by Ficoll-Hypaque. The viral supernatant was collected as described above.

Western blotting (immunoblotting) and endoglycosidase F digestion. To express fusin-HA, HuTK− cells were infected with recombinant vaccinia virus vBD4 encoding fusin-HA at a multiplicity of infection of 10. vSC8 vaccinia virus, which possesses all genes in vBD4 except fusin-HA, was used as a control. Cells were harvested 24 h postinfection by lysis with Triton X-100, and cell debris was removed by centrifugation at high speed in a microcentrifuge. The resulting supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Samples were mixed with SDS-PAGE urea sample buffer, boiled for 5 min, and loaded on an SDS–10% acrylamide gel containing 4 M urea. The gel was transferred onto a polyvinylidene difluoride membrane (Millipore), blocked with Blotto (phosphate-buffered saline [PBS] with 0.1% Tween 20 and 5% dried milk), and probed with the murine monoclonal antibody 12CA5 directed to the HA tag in Blotto for 2 h at room temperature. Bound antibody was detected by incubation with goat anti-mouse horseradish peroxidase (Boehringer Mannheim) (diluted 1:20,000) in Blotto for 45 min at room temperature followed by chemiluminescent detection of horseradish peroxidase (ECL; Amersham).

For detection of N-linked carbohydrates, 12.5 μl of the cell lysate described above was mixed with 12.5 μl of 0.2 M Na phosphate, pH 8.0 (phosphate buffer) containing 0.1% SDS and 0.1% β-mercaptoethanol. Samples were boiled for 5 min before the addition of 25 μl of phosphate buffer and 6 μl of 10% Triton X-100. Treated samples received 4 U of endoglycosidase F (Boehringer Mannheim), while mock-treated samples did not. Samples were digested overnight at 37°C. Samples were analyzed by SDS-PAGE and Western blotting as described above.

Infection assay for HIV-1 entry. pREP8-LESTR (2 μg of plasmid DNA) was transfected into subconfluent CD4$^+$ feline CCCS+L− cells in 24-well tissue culture plates by using calcium phosphate precipitation. The next day the culture medium was changed, and on day 2 posttransfection, cells were infected with cell-free DNase-treated (50 U/ml for 30 min at room temperature) virus stock. Virus infection was synchronized by a 1.5-h adsorption at 4°C followed by a 30-min incubation at 37°C to allow virus entry. The cells were washed twice with cold PBS, growth medium was replaced, and incubation continued at 37°C. Cell lysates for PCR amplification were prepared at various times postinfection by adding 100 μl of PCR lysing buffer (10 mM Tris-Cl [pH 8.3], 50 mM KCl, 0.01% gelatin, 0.45% Tween 20, 0.45% Nonidet P-40) to approximately 2×10^5 cells. Cell samples were incubated overnight at 56°C and then at 95°C for 10 min to deactivate proteinase K.

Nested PCR was performed to amplify the U3 and U5 long terminal repeat (LTR) DNA sequences, and the products were analyzed by electrophoresis on 1.5% agarose gels, transferred to Hybond N+ (Amersham) and probed with a ^{32}P randomly labeled *Kpn*I 608-bp fragment (containing the LTR sequences) from pHIV1lacZ obtained through the AIDS Research and Reference Reagent Program (41). For the first round of PCR amplification, primers U3$^+$57 (5′-CA CACACAAGGCTACTTCCCTG-3′) and U5$^-$596 (5′-GATCTCTAGTTACCA GAGTCAC-3′) were used. For the second round, primers U3$^+$127 (5′-TGGA TGGTGCTTCAAGCTAGTA-3′) and U5$^-$470 (5′-CAGAGAGACCCAGTAC AGGCAA-3′) were used. Species-specific primers CF1 and CF2 (5′-TTTGAC CCCCTGTCATAATATGC-3′ and 5′-TATCGGGGTGGAGTCAAGTAC-3′, respectively) for feline cells were designed to amplify a 184-bp region of the endogenous feline leukemia virus, CF-14 (3).

Gene reporter fusion assay. To quantitate cell-cell fusion events, we utilized the gene reporter fusion assay described by Nussbaum et al. (51). Effector cells expressed T7 polymerase and Env protein, while target cells expressed β-galactosidase under control of the T7 promoter as well as CD4 and fusin. Generally, all proteins were introduced by infection with recombinant vaccinia viruses, except for those cell lines that constitutively express CD4. Vaccinia virus-encoded proteins were produced by infecting cells at a multiplicity of infection of 10 to 20 for 1.5 to 4 h at 37°C. Fusin was introduced into target cells by transfection 1.5 to 2 h postinfection by the calcium phosphate precipitation method. The inoculum was removed, and the cells were washed twice with PBS and then incubated at 32°C overnight in the presence of rifampin. To initiate fusion, target and effector cells were mixed at different ratios in either 96-well plates (when both effector and target cells were in suspension) or in 24-well plates (when adherent target cells were used) at 37°C in the presence of 1-β-D-arabinofuranosylcytosine. To quantitate fusion at different times after initiation, Nonidet P-40 was added to a final concentration of 0.5%, and aliquots of the cell lysates were monitored for β-galactosidase activity by using the colorimetric assay described by Nussbaum et al. (51). In some cases, β-galactosidase was detected by in situ staining (51), or alternatively, syncytium formation was monitored by fixing the cultures in glutaraldehyde-formaldehyde and staining with a solution of 0.5% methylene blue and 0.17% pararosaniline in methanol.

RESULTS

Expression and detection of fusin. Fusin is a 352-amino-acid protein with approximately 33% homology to members of the CC and CXC chemokine receptor families and is predicted to exhibit the topology shown in Fig. 1. Shared structural features

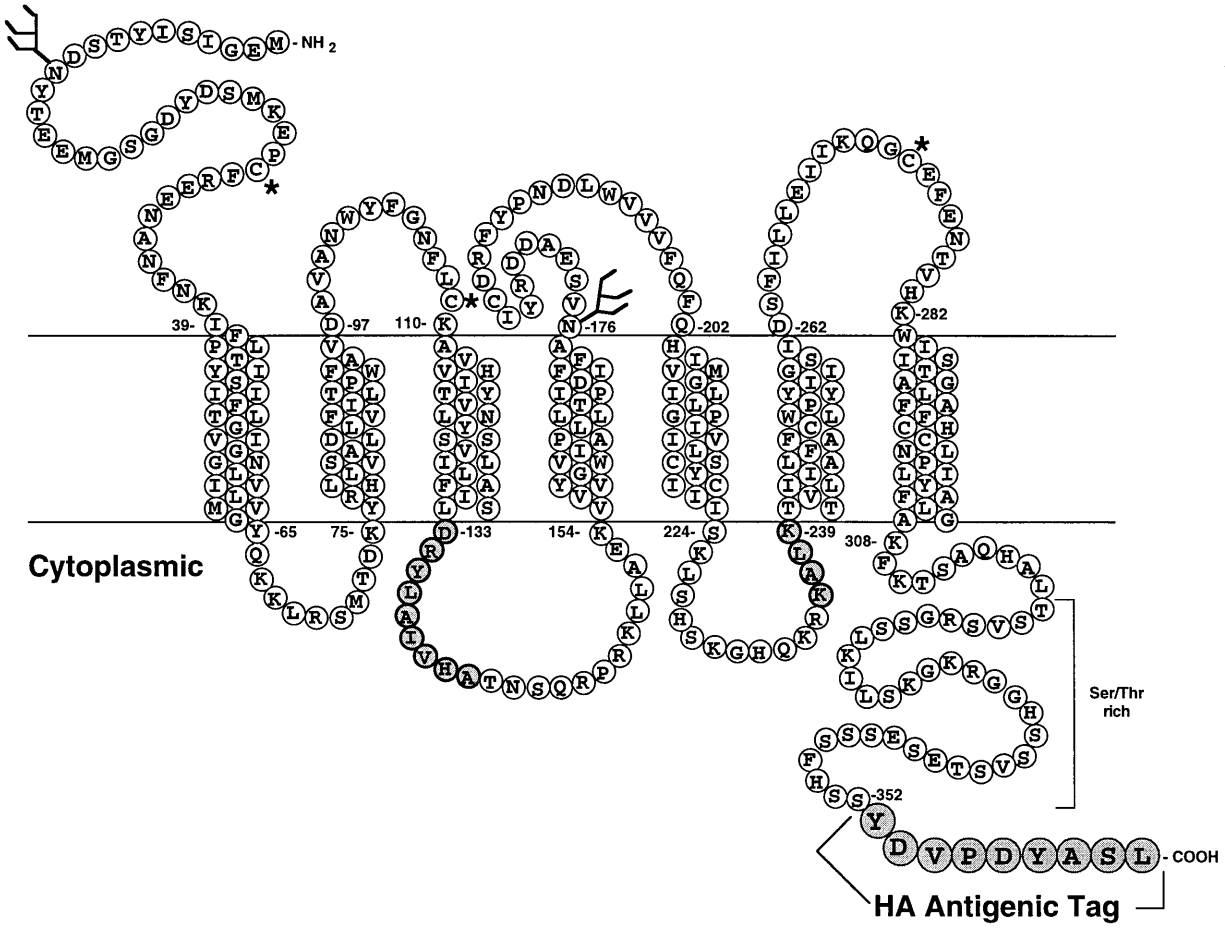

FIG. 1. Schematic representation of fusin. The sequence and proposed membrane topology of fusin are shown. Cys residues in the ectodomain are indicated by an asterisk, and the two potential N-linked carbohydrate addition sites are shown. By analogy with similar receptors, the Cys residues in extracellular loops 1 and 2 are proposed to form a disulfide bond. Conserved regions in cytoplasmic loops characteristic of G-protein-coupled receptors are indicated by shading. An antigenic tag corresponding to a sequence found within influenza virus HA was introduced at the C terminus as indicated.

include seven-transmembrane domains with proline residues in transmembrane domains II, IV, V, VI, and VII, a serine- and threonine-rich C-terminal domain, conserved regions predicted to bind G proteins, two potential N-linked glycosylation sites, and four cysteine residues in the ectodomain (47). By analogy with related molecules, the cysteine residues in extracellular loops 1 and 2 are predicted to form a disulfide bond, as are those in the N-terminal ectodomain and extracellular loop 3 (47).

To express fusin in a variety of cell types, we took advantage of a vaccinia virus vector system. Fusin was cloned into two plasmids, pSC59 and pSC65, thereby placing it under control of the vaccinia virus synthetic early/late promoter (18). These plasmids allowed for transient expression of fusin in two ways: by transfection of the plasmids in conjunction with vaccinia virus infection and by infection with recombinant vaccinia virions. To facilitate detection of fusin, a 9-amino-acid antigenic tag based on the influenza virus HA YDVPDYASL sequence was introduced at the C terminus of fusin. The HA-tagged fusin (fusin-HA) was cloned into pSC65, and a recombinant vaccinia virus was made (vBD4).

To monitor fusin expression, HuTK− cells were infected with vBD4 and incubated overnight. The cells were lysed, and aliquots subjected to SDS-PAGE and Western blotting. Fusin-HA was detected with a monoclonal antibody (12CA5) directed to the HA tag. As shown in Fig. 2 (lanes 1 and 2), fusin-HA was detected by Western blotting as an approximately 50-kDa band. The protein's predicted molecular mass (including the HA tag) is approximately 41 kDa. To determine if fusin contains N-linked carbohydrates, fusin-HA was either mock digested or digested with endoglycosidase F and analyzed by SDS-PAGE and Western blotting. As shown in Fig. 2 (lanes 3 to 5), endoglycosidase F digestion resulted in a 10-kDa shift in mobility from 50 kDa to near its predicted amino acid molecular mass of 41 kDa, indicating that at least one and probably both N-linked consensus sites are utilized (Fig. 1). Identical results were obtained when fusin-HA was immunoprecipitated, digested with endoglycosidase F, and subsequently analyzed by Western blotting (not shown). These results indicate that fusin could be readily expressed and detected by the vaccinia virus expression system and that at least one of two N-linked addition sites is used. Glycosylation of fusin partially confirms the membrane topology depicted in Fig. 1, since there are no potential N-linked glycosylation sites in the proposed cytoplasmic domains.

Fusin supports HIV-1 Env-induced membrane fusion of nonhuman cells. While cells expressing HIV-1 Env form syncytia with most human cells expressing CD4, fusion generally does not occur if the target CD4-bearing cells are nonhuman in origin (2, 5, 13, 16, 17, 40). To determine if fusin could render

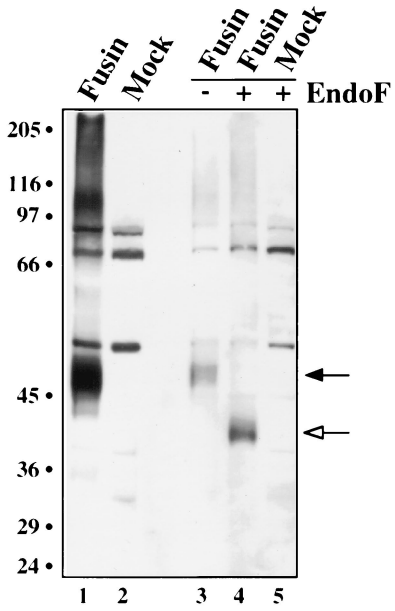

FIG. 2. SDS-PAGE analysis of fusin. HuTK− cells were infected with vBD4 (lanes 1, 3, and 4), a recombinant vaccinia virus that expresses fusin with the HA antigenic tag, or with vSC8 (lanes 2 and 5), a virus that does not contain the fusin-HA gene but is otherwise identical to vBD4. After incubation overnight, the cells were lysed and aliquots were subjected to SDS-PAGE in the presence of 4 M urea. Alternatively, aliquots were mock digested (lane 3) or digested with endoglycosidase F (EndoF) (lanes 4 and 5) overnight prior to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane, and fusin-HA was detected with monoclonal antibody 12CA5. The position of fusin-HA is indicated by the solid arrow, while endoglycosidase F-digested fusin-HA is indicated by the hollow arrow. Samples in lanes 3, 4, and 5 represent 25% of the amount of lysate loaded in lanes 1 and 2. The mobilities of the indicated molecular mass standards (in kilodaltons) are shown to the left of the gel.

such cells permissive for HIV-1 Env-mediated membrane fusion, we utilized a vaccinia virus-based gene reporter fusion assay that has been shown to faithfully recapitulate the salient features of HIV-1 Env-mediated membrane fusion (4, 51). In this assay, murine 3T3 cells were either transfected with pSC59-Fusin or mock transfected. The cells were infected with vCB21r, which encodes β-galactosidase under control of the T7 promoter and activates fusin expression. Some cells were also infected with vCB3, a recombinant virus that expresses huCD4. The 3T3 cells were used as targets for HeLa effector cells that expressed the HIV-1 IIIB Env protein and T7 polymerase as a consequence of infection with recombinant vaccinia viruses. Both target and effector cells were infected overnight, after which they were mixed together and incubated at 37°C for up to 8 h. If fusion occurs, the cytoplasmic contents of the target and effector cells mix, leading to β-galactosidase expression (51). Fusion can therefore be monitored in two ways: visually by scoring for syncytium formation (with or without in situ β-galactosidase staining) and biochemically by measuring β-galactosidase activity.

As shown in Fig. 3B, HeLa cells expressing Env readily formed syncytia with HeLa cells expressing CD4. In situ staining for β-galactosidase activity was performed in order to visualize syncytia more easily. Fusion did not occur when a noncleaved, fusion-inactive form of Env was used (Fig. 3A). When HeLa cells expressing fusion-active Env were incubated with murine 3T3 cells expressing huCD4, fusion did not occur, consistent with previous studies (Fig. 3C). However, fusion was readily detected when the target 3T3 cells expressed both CD4

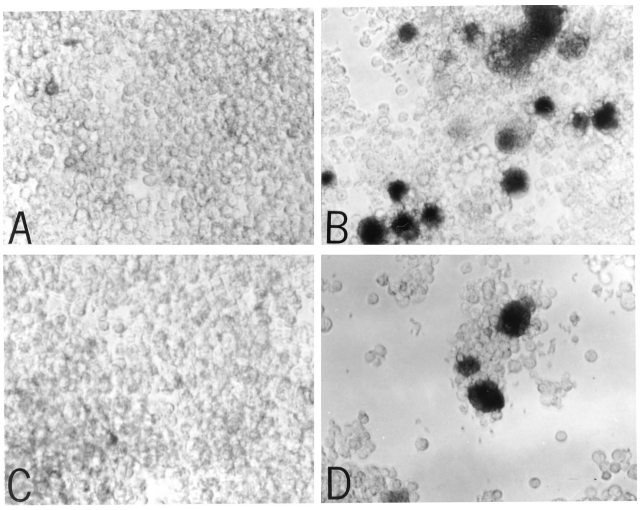

FIG. 3. Fusin supports HIV-1 Env-induced membrane fusion of murine 3T3 cells. HeLa cells infected with vTF1.1 (expressing T7 polymerase) and either vCB16 (expressing a fusion-inactive form of HIV-1 IIIB Env) (A) or vSC60 (expressing HIV-1 IIIB Env) (B, C, and D) were incubated with either HeLa cells (A and B) or murine 3T3 cells (C and D). Both the HeLa and 3T3 cells were infected with vCB21r (encoding *lacZ* under control of the T7 promoter) and vCB3 (expressing huCD4). In addition, the murine cells in panel D were also transfected with pSC59-fusin. After incubation for 8 h, the cells were fixed and stained in situ for β-galactosidase activity.

and fusin (Fig. 3D). To quantitate the extent of fusion, various combinations of murine 3T3 target and HeLa effector cells were lysed at different times after mixing, and the amount of β-galactosidase activity was determined by a colorimetric assay. Figure 4 demonstrates that fusion was detected 3 h after mixing and increased with time. Fusion occurred only when cells expressing the fusion-active form of Env were mixed with cells expressing both huCD4 and fusin. Fusion did not occur

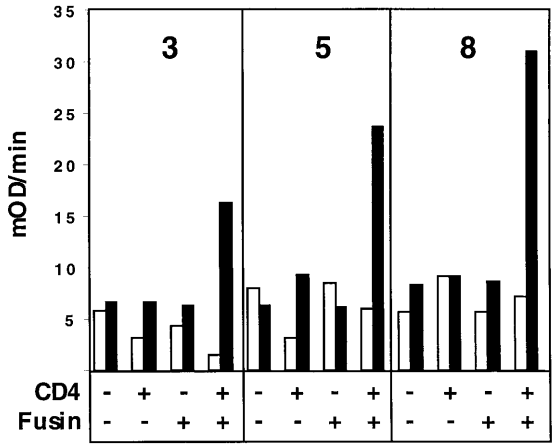

FIG. 4. Time course of fusion. As in Fig. 3, HeLa cells expressing cleaved (fusion-active [black bars]) or uncleaved (fusion inactive [white bars]) IIIB Env and the T7 polymerase were incubated with target 3T3 cells encoding *lacZ* under control of the T7 promoter and expressing the indicated combinations of fusin and huCD4. HuCD4 was introduced by infection with vCB3, while fusin was introduced by transfection. We estimate that 5 to 10% of the target cells were successfully transfected. β-Galactosidase activity was determined at different times after mixing (3, 5, or 8 h, indicated at the top of the panels) and is expressed as milli-optical density units (mOD) per minute.

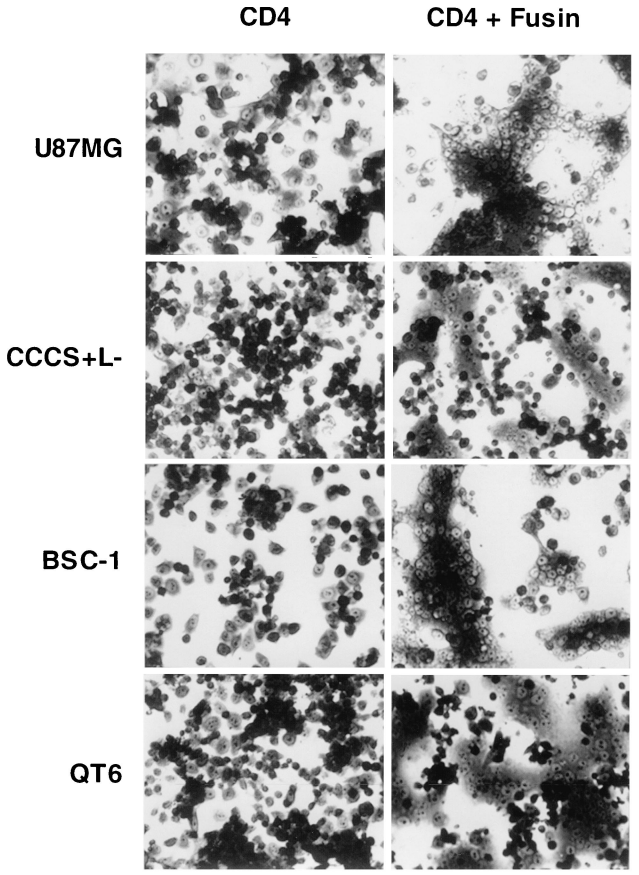

FIG. 5. CD4 and fusin support syncytium formation in different cell types. HeLa cells expressing HIV-1 IIIB Env were mixed with human U87MG, feline CCCS+L−, simian BSC-1, or quail QT6 cells expressing either huCD4 alone or huCD4 and fusin-HA. Both huCD4 and fusin-HA were introduced by use of recombinant vaccinia virus vectors. After 8 h, the cells were fixed and stained with methylene blue.

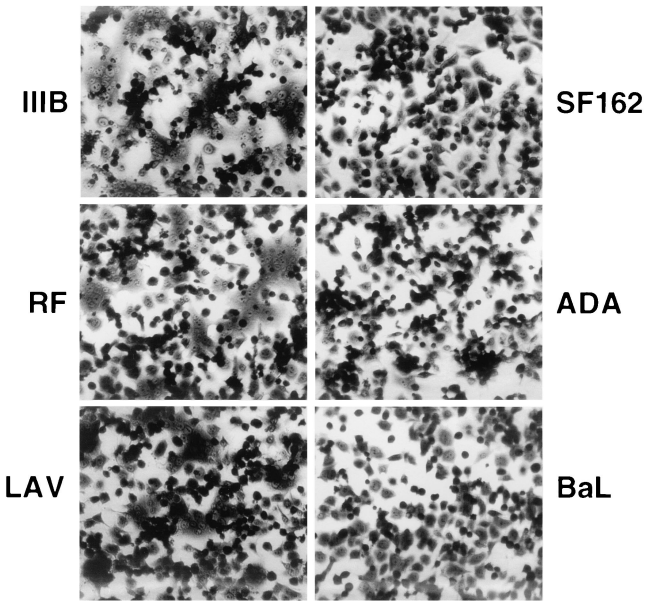

FIG. 6. CD4 and fusin do not support syncytium formation by M-tropic Env proteins. HeLa cells expressing the indicated Env proteins were mixed with feline CCCS+L− cells expressing huCD4 and fusin-HA. The left gels show results with Env proteins from T-tropic viruses, the right gels show results with Env proteins from M-tropic viruses. The cells were fixed and stained with methylene blue 8 h after mixing.

when the target cells expressed either CD4 or fusin alone or when a noncleaved and therefore fusion-inactive form of Env was expressed in the effector cell population (e.g., Fig. 3A).

To determine if the results obtained above were cell type dependent, similar experiments were performed with quail (QT6), feline (CCCS+L−), and simian (BSC-1) cell lines. In addition, a human cell line (U87MG) that is resistant to HIV-induced syncytium formation and infection when expressing CD4 was used (12). huCD4 was introduced into target cells by infection with recombinant vaccinia virus vCB3, while fusin-HA was introduced by infection with vBD4. HeLa cells expressing the HIV-1 IIIB Env protein were used as effector cells. Target and effector cells were incubated together for 8 h, after which the cells were fixed and stained with methylene blue. As shown in Fig. 5, none of the target cells supported syncytium formation when expressing CD4 alone. However, large syncytia formed when both CD4 and fusin were expressed in the target cells. These findings show that coexpression of fusin-HA and huCD4 renders a number of nonhuman cell lines susceptible to HIV-1 Env-induced membrane fusion.

Fusin supports fusion of T-tropic, but not M-tropic, Env proteins. To determine if fusin could serve as an accessory factor for both T- and M-tropic HIV-1 strains, feline CCCS+L− cells expressing huCD4 and fusin were mixed with HeLa effector cells infected with recombinant vaccinia virus vectors that express the Env proteins of the T-tropic strains IIIB, LAV, and RF or the M-tropic strains ADA, Ba-L, and SF-162. The Env proteins expressed by these recombinant viruses have previously been shown to be fusion active (4). As shown in Fig. 6, fusion was readily observed when the effector cells expressed T-tropic Env proteins. Fusion was not observed with the ADA, Ba-L, and SF-162 Env proteins. Thus, while fusin supported fusion by T-tropic HIV-1 strains, it failed to support fusion of the three M-tropic strains tested here.

Fusin supports infection of T-tropic HIV-1 strains. To determine if fusin could render CD4-positive nonhuman cells permissive for HIV-1 infection, we utilized a PCR-based entry assay. $CD4^+$ feline cells (CCCS+L−CD4) were either transfected with pREP8-fusin DNA or mock transfected 48 h prior to virus challenge. At the indicated times postinfection, cell lysates were prepared and subjected to nested PCR analysis using HIV-1 U3 and U5 LTR-specific primers as described in Materials and Methods. An intense band of the predicted size corresponding to the LTR DNA sequence was evident only after PCR amplification of cell lysates prepared from feline cells expressing both fusin and huCD4 and infected with a T-tropic HIV-1 strain, IIIB (HxB2 clone [Fig. 7A]). The very weak band that was sometimes detected for HxB2-infected CD4-positive feline cells not expressing fusin declined over time (Fig. 7A) and so likely represents residual virus inoculum. In contrast, infection of feline cells expressing both fusin and huCD4 with the M-tropic HIV-1 strain Ba-L either resulted in no PCR product or only a very faint band (Fig. 7A). Similar results were obtained with murine PA317-T4 cells (not shown). Amplification with feline specific primers yielded bands of equivalent intensity in all lanes (Fig. 7B), indicating that those samples with weak or no detectable HIV-1-specific sequences contained equal amounts of DNA.

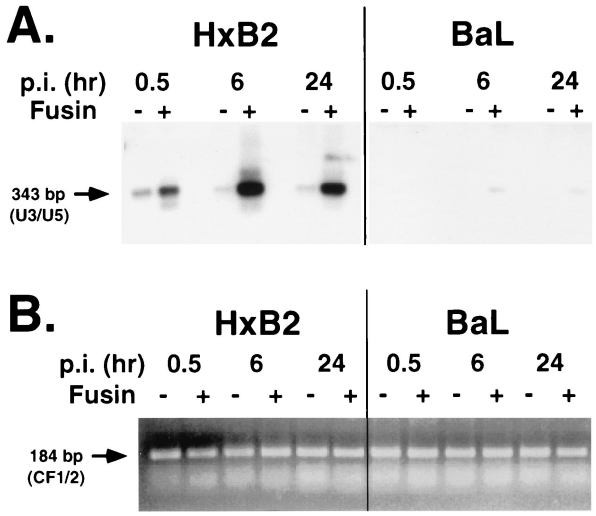

FIG. 7. Fusin supports virus entry. Feline CCCS+L− cells, which constitutively express huCD4, were transfected with pREP8-LESTR as indicated. Two days later, cells were infected with either HIV-1 HxB2 (T-tropic) or Ba-L (M-tropic). Cell lysates were prepared at the indicated times postinfection (p.i.), and PCR amplification of viral U3 and U5 LTR DNA sequences was performed (A). Species-specific primers were used to amplify a 184-bp region of the endogenous feline leukemia virus CF-14 to ensure that equivalent amounts of DNA were used in each sample (B).

DISCUSSION

The entry of HIV-1 into cells is a critical step in the infectious cycle and an important determinant of viral tropism. Binding to a cell surface receptor, such as CD4, must be followed by a conformational change in the Env protein that leads to fusion between the viral envelope and a host cell membrane (for a review, see reference 44). This conformational change is believed to result in the exposure of the N-terminal fusion peptide in the gp41 subunit. While CD4 binding has been shown to induce structural alterations in Env (59), it is clear that CD4 binding in and of itself is not sufficient to trigger the entire sequence of events that lead to membrane fusion and subsequent viral entry. As a consequence, it has been suspected that additional cofactors resident in the plasma membrane of the target cell participate in this process. A number of molecules have been proposed to serve as accessory factors for HIV-1, including LFA (33), CD7 (58), and CD26 (7). While some of these molecules may enhance syncytium formation under certain circumstances, none has proven to be required for infection or cell-cell fusion. In the case of CD26, many investigators have reported that it does not play a role either in virus infection or syncytium formation (1, 6, 8, 21, 38, 52, 62, 63).

In this report, we have shown that fusin, a seven-transmembrane domain glycoprotein, can serve as an accessory factor for a number of T-tropic HIV-1 strains, in agreement with Feng et al. (23). Fusin fulfills the requirements of an accessory factor in that it rendered a number of murine, feline, simian, and quail cell lines, as well as a human cell line resistant to HIV-1 entry, fully permissive for HIV-1 Env-mediated syncytium formation. When fusin was introduced into target cells by infection with a recombinant vaccinia virus rather than by transfection such that all target cells expressed both the cofactor and huCD4, fusion was greatly enhanced, with most cells forming syncytia. Specificity was confirmed in all cases by the finding that fusion occurred only when the effector cells expressed fusion-active Env and when the target cells expressed both fusin and huCD4. In agreement with Feng et al., we found that HIV-1 IIIB could infect cells that coexpressed huCD4 and fusin (23). In addition, we extended their findings by showing that fusin failed to support infection by an M-tropic HIV-1 strain. Taken together, these findings suggest that fusin can serve as a cofactor for T-tropic, but not M-tropic, HIV-1 strains for both Env-mediated syncytium formation and virus infection. A larger panel of virus strains, including viruses from different clades, will have to be examined in order to determine the full range of virus types that can utilize fusin as an entry cofactor.

Fusin is a 352-amino-acid protein that is predicted to contain seven transmembrane domains (22, 32, 35, 39, 50). It is most closely related (approximately 33% homology) to the CXC and CC chemokine receptors, though attempts to demonstrate chemokine binding to fusin have not been successful (32, 35, 39, 50). Thus, fusin is an orphan receptor with no known ligand or function. On the basis of its sequence, its homology with better-characterized receptors, and the fact that one or both of the N-linked glycosylation sites are utilized (Fig. 2), it is likely that fusin exhibits the topology depicted in Fig. 1. Fusin contains an acidic N-terminal ectodomain segment that in other chemokine receptors has been implicated in ligand binding (14, 28, 31). The transmembrane domains are notable in that several contain proline residues, and the intracellular loops contain highly conserved motifs characteristic of G-protein-coupled receptors. The four cysteine residues in the ectodomain of fusin include two in the first and second extracellular loops that are highly conserved and are predicted to form a disulfide bond (61). The presence of an N-linked glycosylation site in the amino-terminal domain is also a common structural motif, and it is utilized in a number of other receptors (47). Finally, the C-terminal domain of fusin, like the chemokine receptors, is rich in serine and threonine residues that could be phosphorylated following ligand binding.

Fusin has been cloned from human monocyte, fetal brain, lung, spleen, and peripheral blood mononuclear cell libraries (22, 32, 35, 39, 50). A homolog isolated from bovine brain bears 93% homology with human fusin (56). Northern (RNA) blot analyses show that fusin is expressed at high levels in B- and T-cell lines, in cell lines derived from the monocytic lineage, and from human peripheral blood lymphocytes, monocytes, and neutrophils (22, 35, 39, 50). High levels of expression are seen in heart and brain tissue, with intermediate levels in colon and liver tissue (22). It is also expressed in HeLa cells (50), which is significant since HeLa cells have been shown to contain the accessory factor for T-tropic viruses (5, 16). Human erythrocytes have also been shown to contain an HIV-1 cofactor, since fusion of human erythrocyte ghosts with murine cells expressing huCD4 makes these cells susceptible for HIV-1 Env-mediated syncytium formation (17). While it is not known if fusin is present in the erythrocyte membrane, it is interesting to note that the Duffy blood group antigen, a seven-transmembrane receptor that bears approximately 20% homology to fusin, is present at relatively high levels and has been shown to bind to RANTES, interleukin-8, and MCP-1 (10, 49, 53). Whether the Duffy antigen can serve as an HIV-1 cofactor is currently being examined. Human cell lines in which fusin mRNA has not been detected include the neuroblastoma cell lines SK-N-MC and LAN5. It is interesting to note that expression of CD4 in SK-N-MC cells does not render them permissive for HIV-1 Env-mediated syncytium formation (39a). Thus, the distribution of fusin is largely consistent with its role as a T-tropic accessory factor—it is present in cells that are permissive for virus infection or membrane fusion, and it is absent in at least one human cell line that is not.

How might fusin participate in HIV-1 entry? The most obvious possibility is that it plays a role similar to that of other

viral accessory factors and binds directly to the Env protein, leading to conformational changes that lead to membrane fusion. The Semliki Forest virus, for example, binds to an as yet unidentified receptor on the cell surface. After the virus is internalized and delivered to endosomes, the acid pH triggers a conformational change in the virus spike protein that enables it to bind to cholesterol (for a review, see reference 36). Cholesterol binding, along with the presence of trace quantities of sphingolipids in the target membrane, is required for the subsequent membrane fusion reaction (36). Insect cells that lack cholesterol are not permissive for Semliki Forest virus infection, but introduction of cholesterol makes them fully susceptible (54). For HIV-1, binding to CD4 is known to trigger conformational changes in Env, but these changes are not sufficient for fusion. However, CD4 binding may allow subsequent binding of Env to fusin, which may then lead to conformational changes that result in exposure of the fusion peptide and membrane fusion. Support for this hypothesis comes from the work of Golding and coworkers, who found that the phorbol ester myristate acetate (PMA) can down-regulate CD4, but not a truncated version of CD4 that lacks its cytoplasmic domain (26, 27). However, PMA-induced down-regulation of the truncated CD4 did occur when cells were incubated with soluble gp120 prior to the addition of PMA. Importantly, this did not occur when tailless CD4 was expressed in nonhuman cell lines. These findings suggest that gp120 binding to CD4 induces conformational changes in either gp120 or CD4 that lead to complex formation with the accessory factor, which itself is down-regulated by PMA (26, 27). Alternatively, fusin may affect the way in which CD4 is presented on the cell surface by affecting CD4 conformation or by leading to patching, since multimeric CD4 binding may be required for fusion to occur (37, 45). The identification of fusin as an accessory factor for the HIV-1 fusion reaction will make it possible to test these and other hypotheses.

While fusin supports T-tropic HIV-1 Env-mediated membrane fusion, it does not appear to serve as an accessory factor for M-tropic HIV-1 strains. Given the similarities between T- and M-tropic Env proteins, it is reasonable to suspect that M-tropic viruses utilize a structurally related molecule. Fusin shares considerable homology with both the CC and CXC chemokine receptors. Two CXC chemokine receptors have been identified, one of which binds interleukin-8, while the other binds interleukin-8 and other CXC chemokines (34, 46). Thus far, four CC chemokine receptors have been identified. Of these, CKR-1, CKR-4, and CKR-5 bind to RANTES, MIP-1α, and MIP-1β, while CKR-2 binds to MCP-1 and MCP-3 (9, 24, 43, 48, 55, 57, 64). Recently, it has been shown that RANTES, MIP-1α, and MIP-1β are the major HIV-1 suppressive factors secreted by $CD8^+$ T cells (15). If certain HIV-1 strains can utilize one or more receptors for these CC chemokines, then the antiviral effects of these chemokines may be mediated in part by the blockade or the down-regulation of the chemokine receptor.

HIV-1, HIV-2, and simian immunodeficiency virus isolates are notable for their diverse cellular tropisms. While some virus strains preferentially infect T cells, others infect macrophages, while others are dual tropic and infect both. Furthermore, certain HIV-1 and HIV-2 strains can infect CD4-negative cells, albeit inefficiently (20, 29). While HIV-1 generally is unable to enter nonhuman cells bearing huCD4, this species restriction is not as strict for HIV-2 and simian immunodeficiency virus (13, 42). Cellular tropism is often determined at the level of virus entry, and evidence has accumulated that the presence or absence of cell-specific accessory factors or cofactors determines whether or not a given virus strain can enter a given CD4-positive cell type. The identification of fusin as a cofactor for a number of T-tropic, but not M-tropic, Env proteins is an important step in understanding viral tropism at the molecular level. Given the diversity of Env protein sequences from different viral strains and the large number of seven-transmembrane domain proteins that bear significant homology to fusin, it will not be surprising if other proteins in this class participate in the entry of different HIV-1, HIV-2, and simian immunodeficiency strains.

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