Distinct Requirements for HIV-Cell Fusion and HIV-mediated Cell-Cell Fusion*

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Background: Determinants of HIV-1 fusion with the plasma membrane versus endosomal membrane are unknown.

Results: Unlike HIV-cell fusion, HIV-mediated fusion of the plasma membranes of adjacent cells occurred with much lower probability and was actin-dependent.

Conclusion: Distinct regulation of HIV-mediated cell-cell fusion and virus-cell fusion indicates that the latter events may occur in endosomes.

Significance: These results shed light on the HIV entry routes.

Whether HIV-1 enters cells by fusing with the plasma membrane or with endosomes is a subject of active debate. The ability of HIV-1 to mediate fusion between adjacent cells, a process referred to as “fusion-from-without” (FFWO), shows that this virus can fuse with the plasma membrane. To compare FFWO occurring at the cell surface with HIV-cell fusion through a conventional entry route, we designed an experimental approach that enabled the measurements of both processes in the same sample. The following key differences were observed. First, a very small fraction of viruses fusing with target cells participated in FFWO. Second, whereas HIV-1 fusion with adherent cells was insensitive to actin inhibitors, post-CD4/coreceptor binding steps during FFWO were abrogated. A partial dependence of HIV-cell fusion on actin remodeling was observed in CD4+ T cells, but this effect appeared to be due to the actin dependence of virus uptake. Third, deletion of the cytoplasmic tail of HIV-1 gp41 dramatically enhanced the ability of the virus to promote FFWO, while having a modest effect on virus-cell fusion. Distinct efficiencies and actin dependences of FFWO versus HIV-cell fusion are consistent with the notion that, except for a minor fraction of particles that mediate fusion between the plasma membranes of adjacent cells, HIV-1 enters through an endocytic pathway. We surmise, however, that cell-cell contacts enabling HIV-1 fusion with the plasma membrane could be favored at the sites of high density of target cells, such as lymph nodes.

HIV-1 envelope glycoprotein (Env)4 initiates the release of the nucleocapsid into the cytosol by fusing the viral membrane with the host cell membrane, a process that is initiated upon the sequential binding of Env to CD4 and coreceptors, CCR5 or CXCR4. The assembly of ternary Env-CD4-coreceptor complexes triggers refolding of the transmembrane gp41 subunit into the final 6-helix bundle structure, which ultimately leads to the merger of viral and cellular membranes.

Although the mechanism of Env-mediated fusion is reasonably well understood (1–3), the sites of HIV-1 entry into cells remain controversial (4, 5). The widely held view that HIV-1 directly fuses with the plasma membrane (6–11) has been challenged by several studies showing pH-independent endocytic entry of this virus into different cell types, including primary macrophages (12–23). We have previously provided functional evidence supporting HIV-1 entry through endocytosis in adherent cells and CD4+ T cell lines (13). Single particle imaging revealed that HIV-1 fusion with the plasma membrane (PM) did not progress beyond a lipid mixing (hemifusion) stage (13), implying that this virus failed to undergo full fusion with the PM. By contrast, single virus fusion with endosomes was readily detected. Attempts to redirect HIV-1 fusion to the cell surface by blocking its uptake were unsuccessful (12). However, a recent study of HIV-1 fusion with lymphoid cells has concluded that endocytosis does not contribute to productive entry (24). Moreover, the ability of HIV-1 Env expressed on the cell surface to promote fusion with target cells (25–27) as well as the ability of HIV-1 particles to mediate fusion between adjacent cells (a phenomenon commonly referred to as “fusion-from-without”; FFWO) (28) demonstrate that this virus can fuse with the PM.

Virions responsible for FFWO fuse with the plasma membranes of adjacent cells, providing a unique opportunity to study HIV-1 fusion occurring exclusively at the cell surface. To compare the entry and fusion of “free” HIV-1 particles with virus-mediated cell-cell fusion, we developed a dual-readout assay that enabled the measurement of both processes in the same sample. Our data show that FFWO occurs much less fre-
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quently than HIV-cell fusion and that FFWO, but not the fusion of free viruses with adherent cells, was potently inhibited by actin-disrupting drugs. Intact actin was required for the late stages of HIV-mediated cell-cell fusion downstream of CD4 and coreceptor engagement. Deletion of the gp41 cytoplasmic tail (CT) dramatically enhanced FFWO while only modestly affecting virus-cell fusion. The marked differences in actin- and gp41 CT dependence of FFWO involving the PM versus free virus are in line with the notion that HIV-1 normally enters adherent cells via endocytosis (12, 13).

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Reagents—Human embryonic kidney 293T/17 cells (referred to as 293T cells) were obtained from the ATCC (Manassas, VA). 293T-DSP(1–7) cells, constitutively expressing the DSP(1–7) fragment were described previously (29). The NP2 glcoma cell lines expressing CXCR4 and/or CD4 have been described previously (30). Their derivatives, NP2/CD4/CXCR4/DSP(1–7) and NP2/CD4/CXCR4/DSP(8–11), constitutively express the DSP(1–7) or DSP(-11) fragments (hereafter abbreviated as DSP-1 and DSP-2, respectively (29)). Human lymphoid CEM.NKR-CCR5-Luc cells (donated by Drs. J. Moore and C. Spenlehauer (31)) were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health.

The pCAGGS plasmid harboring the full-length HXB2 Env was provided by Dr. J. Binley (Torrey Pines Institute, CA) (32). Mature or immature HIV-1 particles bearing the full-length or cytoplasmic tail-deleted Env were produced using the pIIINL4env and pIIINL4envCTdel-144 plasmids kindly provided by Drs. E. Freed (33). The CXCR4-tropic HIV-1 molecular clone pR8 lacking env (pR8ΔEnv) was obtained from Dr. D. Trono (University of Geneva, Switzerland). The pcRev plasmid (34) and the pMM310 plasmid expressing BlaM-Vpr (35) were obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health.

Phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) was from Sigma. Hanks’ balanced salt solution with calcium and magnesium (HBSS), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Cellstripper, minimum essential Eagle’s medium, and RPMI 1640 medium were from Cellgro (Manassas, VA). Polyfect was obtained from Qiagen (Valencia, CA), and EnduRen™ was from Promega (Madison, WI). The BMS-806 compound (36, 37) was synthesized by ChemPacific Corp. (Baltimore, MD). AMD3100 (38) and Pronase were purchased from Sigma. The C52L recombinant peptide (39) was a kind gift from Dr. Min Lu (University of New Jersey). Jasplakinolide (Jasp), latrunculin A (LatA), cytochalasin D (CyD), nocodazole, and paclitaxel were purchased from Sigma. Blebbistatin and nitolitinib were from Calbiochem and LC Laboratories (Boston, MA), respectively.

Virus Production—For HIV-1 pseudovirus production, pCAGGS-HXB2-gp160 (3 μg), pR8ΔEnv (2 μg), pMM310 (3 μg), and pcRev (1 μg) plasmids were mixed with 300 μl of DMEM and 80 μl of Polyfect. The mixture was incubated for 10 min at room temperature, diluted to 1 ml with DMEM with 10% FBS, and added to a 50–70% confluent monolayer of 293T cells in a 100-mm dish. Twelve hours after transfection, the medium was replaced with 6 ml of warm DMEM with 10% FBS, without phenol red. The plate was further incubated for 36 h at 37 °C in a 5% CO2 incubator. Cell culture medium was collected, passed through a 0.45-μm filter, layered on 20% sucrose in PBS, and subjected to ultracentrifugation in an SW41 swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 100,000 × g for 2 h at 4 °C. The pellet was resuspended in phenol red-free DMEM, aliquoted, and stored at −80 °C. Virus titer was determined by a β-galactosidase assay, using TZM-bl cells, as described previously (40). For production of pseudoviruses bearing the full-length or cytoplasmic tail-deleted HIV-1 Env, 293T cells were transfected with 3 μg of pIIINL4env or pIIINL4envCTdel-144 plasmid, 2 μg of pR8ΔEnv, 3 μg of pMM310, and 1 μg of pcRev.

ELISA and Western Blotting—The amount of HIV-1 p24 in virus preparations was determined by ELISA, as described previously (41, 42). For Western blotting, concentrated viral samples containing equal amounts of p24 were boiled for 10 min at 95 °C in a sample buffer (Bio-Rad) supplemented with 5% β-mercaptoethanol and loaded onto a 10% polyacrylamide gel (Bio-Rad). Separated proteins were transferred to a nitrocellulose membrane, blocked with 10% Blotting-grade Blocker (Bio-Rad) for 1 h at room temperature, and identified using anti-gp120 antibodies (Fitzgerald Industries, Acton, MA), anti-gp41 Chessy8, or anti-HIV sera (both from the AIDS Reference Reagent Program, National Institutes of Health) in 5% Blotting-grade Blocker at 4 °C overnight. The resulting bands were visualized with HRP-conjugated anti-mouse antibody (GE Healthcare) or HRP-conjugated Protein G (Bio-Rad) and the chemiluminescence reagent (GE Healthcare), using Chem-Doc Imager (Bio-Rad).

Immunofluorescence Staining—DSP-1 or DSP-2 cells grown to near confluency on 8-well chamber coverslips were washed twice with PBS and permeabilized with 1.0% Triton X-100 in PBS for 4 min at room temperature. The detergent was removed by washing, and cells were incubated with 1 unit/well of AlexaFluor488-phalloidin (Invitrogen, 200 units/ml stock in methanol) for 20 min at room temperature. After removing uninorporated phalloidin, cell nuclei were stained with Hoechst-33342 (Invitrogen).

Cell Viability Assay—DSP-1/DSP-2 cells were spinoculated with the HXB2 pseudoviruses at 2095 °C × g for 30 min, washed, incubated with 3 μM of each actin inhibitor or DMSO in HBSS on ice, and shifted to 37 °C for 90 min to allow fusion. At the end of incubation, the cells were chilled on ice, mixed with 50 μl of DMEM, 10% FBS and 10 μl of CellTiter 96 Aqueous Reagent (Promega), and further incubated at 37 °C for 90 min. The absorbance at 490 nm was recorded using Synergy HT fluorescence plate reader.

Cell-Cell Fusion—293T cells stably expressing DSP-1 were transfected with 3 μg of pCAGGS-HXB2-Env and 1 μg of pcRev using the Polyfect reagent. In parallel, DSP-2 cells were stripped from plates and added to the Matrix 96-well black-well clear-bottom plate (Thermo Scientific, Waltham, MA) coated with collagen. The next day, 293T-DSP-1-Env cells were preincubated with 40 μM EnduRen™ in HBSS for 2 h and overlaid onto DSP-2 cells at a 1:1 ratio. Cell fusion was initiated by raising the temperature to 37 °C, and the luciferase activity was measured with a TopCount NXT plate reader (PerkinElmer Life Sciences).
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**Virus Endocytosis Assay**—To measure HIV-1 uptake by DSP-2, cells cultured on a collagen-coated 96-well strip plate (1·10⁵ cells per well) were pretreated with cytoskeleton inhibitors in HBSS, 10% FBS for 20 min at 37 °C and centrifuged with HXB2 pseudoviruses containing BlaM-Vpr (m.o.i. = 1) at 2095 × g at 4 °C for 30 min. Unbound viruses were washed out, and the samples were incubated in HBSS, 10% FBS in the presence or absence of inhibitors for 90 min at 37 °C. Viruses remaining on the cell surface were removed by treating with 2 mg/ml Pronase (Sigma) for 10 min on ice. Cells were washed with HBSS, 10% FBS and lysed, and the endocytosed virus was determined by p24 ELISA, as described above.

A similar strategy was employed to measure HIV-1 uptake by CEM.NKR-CCR5.Luc cells. Briefly, ∼1·10⁵ cells were dispensed into each well of U-bottom 96-well plates (Corning Costar), centrifuged at −900 × g for 3 min at room temperature, and resuspended in culture media containing or lacking the indicated inhibitors. Cells were then incubated for 30 min at 37 °C and pelleted by centrifugation, as described above. BlaM-Vpr-containing HXB2 pseudoviruses were added to cells at m.o.i. = 2 and centrifuged at 2095 × g at 4 °C for 30 min. Cells were washed extensively and incubated in complete growth medium in the presence or absence of inhibitors for 2 h at 37 °C. Surface-accessible virions were removed by treating with 0.25% trypsin (Sigma) for 4 min at room temperature. Cells were washed with the growth medium and lysed, and the amount of internalized viruses was determined by p24 ELISA, as described above. The fraction of internalized viruses was determined by normalizing to the total amount of p24 in the trypsin-untreated control samples.

**Virus-Cell Fusion**—The virus-cell fusion measurements were performed as described previously (12, 13). Briefly, DSP-1 and DSP-2 cells were co-cultured at a 1:1 ratio on a collagen-coated 96-well strip plate. Pseudoviruses (m.o.i. = 1, unless indicated otherwise) were prebound to cells by centrifugation at 2095 × g for 30 min at 4 °C. Unbound viruses were washed away, and fusion was initiated by shifting the samples to 37 °C. The fusion reaction was stopped at the indicated time points by adding the fusion inhibitors or by placing cells on ice (referred to as the temperature block). Cells were then loaded with the fluorescent CCF4-AM substrate (Invitrogen) and incubated overnight at 37 °C, and the resulting luciferase signal was monitored at different time points, using TopCount NXT reader (PerkinElmer Life Sciences). After reading the cell-cell fusion signal, the media were removed; cells were loaded with the fluorescent CCF4-AM substrate (Invitrogen), and the BlaM signal was measured after an overnight incubation at 12 °C, as described above. To assess the effects of the cytoskeleton inhibitors, DSP-2 cells were preincubated with 40 μM of the cell-permeable renilla luciferase substrate EnzuRen™ in HBSS, 10% FBS for 2 h, centrifuged with viruses for 30 min at 4 °C (2095 × g) to allow virus binding, and washed once with ice-cold HBSS, 10% FBS. FFWO was initiated by incubation at 37 °C, and the resulting luciferase signal was monitored at different time points, using TopCount NXT reader (PerkinElmer Life Sciences). After reading the cell-cell fusion signal, the media were removed; cells were loaded with the fluorescent CCF4-AM substrate (Invitrogen), and the BlaM signal was measured after an overnight incubation at 12 °C, as described above. To assess the effect of spinoculation of HIV-1 fusion, control experiments were performed using the following virus prebinding protocol. Confluent DSP-2 cells (∼1·10⁵ cells/well) were preloaded with EnduRen™ for 2 h at 37 °C. Samples were briefly chilled on ice and incubated with HXB2 pseudovirus (m.o.i. = 3) for 90 min at 20 °C, either in the presence or in the absence of 1 μM C52L. Next, nonenzymatically harvested DSP-1 cells were overlaid on top of DSP-2 cells and incubated at 20 °C for an additional hour. During the last 5 min of incubation, 3 μM LatA or a respective volume of DMSO was added, and samples were incubated at 37 °C for 2 h to allow fusion. After measuring the luciferase signal, samples were placed on ice; the media were removed; the CCF4-AM substrate was added, and the BlaM activity was measured after an overnight incubation.

FFWO between CEM.NKR-CCR5.Luc cells was measured by dividing the cell suspension in half and labeling with either 2 μM CellTracker™ Orange (CMRA) or 1 μM CellTracker™ Green (CMFDA, Molecular Probes). Cells were mixed at a 1:1 ratio, and 1·6·10⁵ cells were dispensed in each well of a U-bottom 96-well plate. Cells were then treated with cytoskeleton inhibitors in growth media for 20 min at 37 °C. The inhibitors were removed, and the HXB2 pseudoviruses (m.o.i. = 10) were bound by centrifugation at 2095 × g at 4 °C for 30 min. Unbound viruses were washed out, and the samples were incubated in growth media in the presence or absence of inhibitors for 3 h at 37 °C. The samples were then placed on ice, washed with cold PBS, and transferred onto poly-1-lysine-coated eight-chamber slides (Lab-Tek, Nunc). Cells were allowed to attach.
to slides for 5 min at 4 °C and visualized on an Olympus IX-81 microscope equipped with an EM-CCD camera (Hamamatsu, C9100-12). The fraction of fused cells (positive for both green and orange markers) was determined by ImageJ (National Institutes of Health).

To create a temperature-arrested stage (TAS) of fusion, HIV-1 pseudoviruses (m.o.i. = 3) were prebound to EnduRen™-treated DSP-2 cells by spinoculation, as described above. DSP-1 cells were nonenzymatically detached using Cellstripper and overlaid onto DSP-2 cells at 2:1 ratio, and the samples were incubated at 20 °C for 3 h. Actin inhibitors were added during the last 5 min of incubation at 20 °C, prior to shifting to 37 °C. Alternatively, fully inhibitory concentrations of inhibitors of CD4 binding (BMS-806, 40 μM), CXCR4 binding (AMD3100, 90 μM), or gp41 6-helix bundle formation (C52L, 1 μM) were added immediately prior to raising the temperature. After adding respective inhibitors, cells were incubated at 37 °C for 2 h, and the resulting luciferase and BlaM signals were measured, as described above.

Efficiency of Virus-Cell Fusion Versus FFWO—To estimate the efficiency of virus-cell fusion and FFWO, parallel confluent co-cultures of DSP-1 and DSP-2 cells (1:1 ratio) were spinoculated with a low m.o.i. (≤0.03) of virus, as described above. The samples were washed and incubated at 37 °C for 2 h to allow fusion (for a negative control, incubation was done in the presence of 1 μM C52L). The FFWO samples were further incubated at 37 °C for 4 h to allow the maturation of recombined split GFP in fused cells (in the presence 1 μM C52L to block additional fusion events). For the virus-cell fusion measurements, samples were placed on ice immediately after a 2-h incubation at 37 °C, loaded with the CCF4-AM substrate, and incubated overnight at 12 °C. The BlaM- and GFP-positive cells were visualized on a Zeiss LSM780 confocal microscope or an Olympus IX-81 microscope. Identification of positive cells after removing the fluorescence background was done with the Velocity software (PerkinElmer Life Sciences).

RESULTS

Measurements of Virus-Cell Fusion and Virus-mediated Cell-Cell Fusion in the Same Sample—To compare HIV-induced cell-cell fusion (FFWO) to HIV-cell fusion in the same sample, we developed a dual-readout assay. FFWO was measured using the dual-split protein (DSP) assay, which is based on functional recombination of complementary domains of a dual-split luciferase-GFP chimera expressed in fusing partners (29, 43, 44). The DSP assay detects fusion of cells expressing DSP-1 (the N-terminal fragments of split GFP and split Renilla luciferase (linked together)) with the population of cells expressing DSP-2 (the C-terminal fragments of these reporter proteins, Fig. 1A, top). Upon cell-cell fusion, DSP-1 and DSP-2 fragments expressed in different cells associate with each other, generating functional GFP and Renilla luciferase proteins, as evidenced by the appearance of fluorescence and luminescence signals, respectively. This assay has been successfully used for studies of HIV-1 Env-mediated cell-cell fusion (29, 43, 44). Following the FFWO measurement in cells loaded with a cell-permeable Renilla luciferase substrate, the extent of HIV-cell fusion was determined in the same sample using the β-lactamase (BlaM) assay, as described previously (13, 45). Briefly, the activity of the virus-incorporated BlaM-Vpr chimera released into the cytosol as a result of fusion was determined based on a green-to-blue shift in fluorescence of cells loaded with a BlaM substrate (Fig. 1A, bottom).

To measure FFWO mediated by HIV-1 pseudoviruses, we utilized two NP2 glioma cell lines (30) stably expressing CD4 and CXCR4 and either DSP-1 or DSP-2 split proteins (NP2.CD4.CXCR4.DSP-1 and NP2.CD4.CXCR4.DSP-2, respectively) (29). For brevity, we will refer to these cells as DSP-1 and DSP-2 cells. Both DSP-1 and DSP-2 cells supported efficient HIV-1 pseudovirus fusion, as determined by the BlaM assay (Fig. 1B and data not shown).

HIV-1 Fusion with NP2-derived Cells Likely Occurs after Virus Uptake—Before comparing virus-cell fusion and FFWO, we sought to determine whether HIV-1 enters NP2-derived cells through endocytosis. We have previously designed a BlaM-based kinetic assay to deduce the route of HIV-1 entry into cells (13). This strategy relies on the kinetic difference between the virus escape from the membrane-impermeant fusion inhibitor and from low temperature applied after different times of virus-cell coincubation. The rationale for this strategy is as follows. Virus fusion at the cell surface should result in the identical kinetics of escape from both a peptide inhibitor and low temperature, whereas virus uptake and fusion with endosomes should be manifested in delayed escape from the temperature block compared with a peptide inhibitor.

Measurements of HIV-1 fusion with a co-culture of DSP-1 and DSP-2 cells revealed that the kinetics of virus escape from the cold block was slower than escape from a fully inhibitory concentration of the gp41-derived C52L peptide inhibitor (Fig. 1B). Particles that acquired resistance to C52L became resistant to the low temperature block after ~10 min. This result suggests that, similar to other cell types (12, 13), HXB2 Env-pseudotyped particles enter NP2-derived cells via endocytosis, which renders the virus resistant to a peptide inhibitor. Subsequent viral fusion with intracellular compartments confers resistance to cold.

HIV-mediated Cell-Cell Fusion Is a Low Probability Event—We measured FFWO using either of the two formats referred to as co-culture and overlay (Fig. 1D). The first format involves overnight co-culture of DSP-1 and DSP-2 cells at 1:1 ratio to form a confluent monolayer. Pseudoviruses are then spinoculated onto cells in the cold, and virus-cell and cell-cell fusion are initiated by shifting to 37 °C. HIV-1 pseudoviruses promoted fusion between DSP-1 and DSP-2 cells, as evidenced by a robust increase in the luciferase activity compared with the background signal from cells incubated in the absence of virus (Fig. 1C). Additional controls using HIV-1 fusion inhibitors, BMS-806 (post-CD4 binding (36, 37)), AMD3100 (CXCR4 binding (38)), and C52L peptide (gp41 6-helix bundle formation (39)) also exhibited background-level signals. Also, no detectable luciferase signal above the background was observed for a mismatched cell pair, CD4/CXCR4/DSP-1 and CD4/DSP-2 cells lacking CXCR4 (Fig. 1C). These results show that the DSP assay faithfully reports cell-cell fusion mediated by HIV-1 pseudoviruses. As documented previously (12, 13), the same concentrations of fusion inhibitors fully blocked the BlaM signal in NP1 and NP2 cells (data not shown and Fig. 1B).
To compare the relative efficiencies of HIV-cell fusion and FFWO, we modified the above assays to enable quantification of these two fusion processes using independent single cell-based readouts. The extent of FFWO for varied viral inputs was evaluated based on the number of GFP-positive cells resulting from functional recombination of the split fragments (Fig. 2A, lower panel). In parallel samples, the efficiency of HIV-cell fusion was determined by measuring the fraction of cells posi-
tive for BlaM-Vpr, as evidenced by a blue shift in fluorescence of cells loaded with the BlaM substrate (Fig. 2A, top). To ensure that most GFP- and BlaM-positive cells reflect single cell-cell or virus-cell fusion events, we reduced the virus input to yield a small fraction (~25%) of positive cells. Under identical experimental conditions that minimized the occurrence of multiple virus-cell or cell-cell fusion events; an ~50-fold larger number of cells were positive for BlaM compared with GFP (Fig. 2B). Even after correcting for the lack of GFP signal following the “homotypic” DSP-1 to DSP-1 and DSP-2 to DSP-2 fusion, it is clear that FFWO occurs with much lower probability than virus-cell fusion (given the 1:1 ratio of these cells, the actual FFWO efficiency could be 2-fold greater than the measured efficiency). The low probability of FFWO is not completely unexpected, considering that viruses inducing cell-cell fusion should (i) bind to receptors on two adjacent cell membranes and (ii) create not one but at least two fusion pores to fuse adjacent cells. These highly fusogenic particles likely constitute a minor fraction of HIV-1 pseudoviruses. The low apparent extent of HIV-1 fusion at the cell surface argues against an alternative mechanism of FFWO, whereby functional Env glycoproteins released into the PM of one cell through viral fusion induce fusion with an adjacent cell.

Cell-Cell but Not Virus-Cell Fusion Relies on an Intact Actin Network—We next assessed the role of actin in HIV-cell fusion and FFWO, using actin-depolymerizing drugs LatA and CytD as well as the actin filament-stabilizing drug Jasp. Disruption of actin filaments by these drugs was verified by staining with fluorescent phalloidin (Fig. 3A). Measurements of virus-cell fusion and FFWO in the presence of actin inhibitors were performed using the overlay configuration (Fig. 1D). Here, viruses were prebound to a confluent monolayer of DSP-2 cells in the cold, washed, and overlaid with an excess of DSP-1 cells harvested from culture dishes with a nonenzymatic buffer. A full coverage of adherent cells by the overlaid fusion partners ensures a gravity-driven physical contact between cells and viruses (Fig. 1D), even if the adherent cells shrink in the presence of actin inhibitors.

We found that actin-disrupting drugs did not inhibit HIV-1 uptake (Fig. 3B) or the virus-cell fusion (Fig. 3C), whereas the HIV-1 fusion inhibitors, BMS-806, AMD3100, and C52L, effectively blocked fusion in this configuration (data not shown).
In stark contrast, all three actin inhibitors virtually abrogated the DSP signal originating from virus-mediated cell-cell fusion (Fig. 3C) without affecting cell viability (Fig. 3B). LatA and CytD nearly fully blocked FFWO at 1 μM. Consistent with the dose-dependent effect of Jasp on the morphology of actin filaments (Fig. 3A), this drug less potently attenuated FFWO at lower doses but reduced the extent of cell-cell fusion by 80% at 3 μM.

In control experiments, fusion of 293T cells expressing DSP-1 and HXB2 Env with target DSP-2 cells was also blocked by actin inhibitors (Fig. 3D). Thus, in line with the previous reports (26, 46–48), both HIV-1 Env-mediated cell-cell fusion and FFWO require intact actin.

We then asked whether both fusing partners must have intact actin to support FFWO. For this purpose, either adherent
DSP-2 cells or suspended DSP-1 cells were pretreated with 3 μM LatA or Jasp for 30 min at 37 °C and washed to remove the drug prior to co-culture. HIV-1 pseudoviruses were then spinoculated onto DSP-2 cells and overlaid with DSP-1 cells (Fig. 1D) prior to raising the temperature. Regardless of which fusion partner was pretreated with actin inhibitors, the extent of FFWO was markedly reduced, albeit less potently than when both cell lines were exposed to drugs (Fig. 3E). These asymmetric pretreatment experiments show that FFWO requires intact actin in both fusing cells. Importantly, the fact that pretreatment of suspended DSP-1 cells followed by incubation with adherent cells/viruses in the absence of drugs was sufficient to inhibit FFWO rules out the possibility that disruption of cell-cell contacts due to cell shrinkage was the reason for the compromised FFWO activity seen in Fig. 3C.

To promote virus binding to cells, the above experimental protocols employed a brief (30 min) centrifugation of cells with viruses (referred to as spinoculation (49)). However, a study by Guo et al. (50) has elegantly shown that spinoculation of CD4+ T cells enhances HIV-1 infection by inducing signaling and actin remodeling and that Jasp pretreatment reduces the effect of spinoculation. Although the authors did not examine a direct effect of spinoculation on viral fusion or infection in adherent cells, their results suggest the possibility that diminished FFWO in the presence of actin inhibitors could be related to the spinoculation step employed in our experiments. Considering that FFWO but not HIV-cell fusion was affected by actin inhibitors in the same sample (Fig. 3C), this possibility appeared unlikely. We nonetheless conducted control experiments to rule out a selective effect of spinoculation on FFWO. Adherent DSP-2 cells were preincubated with pseudoviruses at reduced temperature, overlaid with DSP-1 cells, and incubated at 37 °C to allow fusion. Under these conditions, HIV-cell fusion was only modestly affected by LatA, whereas FFWO was abrogated (Fig. 4A), demonstrating that centrifugal force was not responsible for the selective effect of actin inhibitors on FFWO.

Microtubules and Myosin II Contribute to FFWO—We and others have shown that HIV-1 fused efficiently with target cells pretreated with microtubule depolymerizing drugs (13, 51). To assess the role of microtubules in FFWO, DSP-1 and DSP-2 cells were pretreated with nocodazole (to depolymerize microtubules) or with paclitaxel (to stabilize microtubules), and the ability of HIV-1 pseudoparticles to mediate cell-cell fusion was evaluated. Because these drugs did not cause cell shrinkage (data not shown), experiments were carried out in a co-culture format (Fig. 1D). At the highest concentration tested, nocodazole, paclitaxel, and blebbistatin, a nonmuscle myosin II inhibitor, reduced FFWO by 40–60% (Fig. 4B). By contrast, neither microtubule nor myosin II inhibitors affected HIV-cell fusion, as measured in the same samples by the BlaM assay. In control experiments, nilotinib, a selective Abl kinase inhibitor (46), did not diminish FFWO (Fig. 4B). Neither of the tested compounds affected cell viability (data not shown).

To rule out an adverse effect of inhibitors on the assembly of functional DSP proteins in fused cells, we carried out an in vitro DSP assembly assay. Freshly prepared lysates of DSP-1 and DSP-2 cells were mixed, and the resulting luciferase activity was measured after incubation at 25 °C for 10 min. The luciferase activity in vitro

![Figure 4. FFWO dependence on cytoskeleton.](image-url)

**A.** Effects of LatA on FFWO (DSP assay) and HIV-cell fusion (BlaM assay) in control experiments in which HXB2 pseudoviruses were prebound to cells without spinoculation. DSP-2 cells were allowed to bind viruses for 1.5 h at 20 °C (to minimize virus uptake), overlaid with DSP-1 cells, further incubated at 20 °C, and shifted to 37 °C to allow fusion. Data are means ± S.E. from two independent triplicate experiments. **B.** Effects of 10 μM nocodazole (Nocod), paclitaxel (Pacl), blebbistatin (Blebb), and 1.5 μM nilotinib (NIL) on FFWO between DSP-1 and DSP-2 cells mediated by HXB2 pseudoviruses and on pseudovirus fusion with DSP-1/DSP-2 cells (measured by the DSP and BlaM assays, respectively). FFWO experiments were performed in the cell co-culture format, as illustrated in Fig. 1D. Data points are means ± S.E. from two independent triplicate experiments. ***,** p < 0.001. C, effect of cytoskeleton inhibitors on the ability of DSP-1 and DSP-2 fragments to form a functional DSP protein in vitro. Lysates of DSP-1 and DSP-2 cells were prepared and mixed in the absence (DMSO) or in the presence of inhibitors. The luciferase activity was measured after incubation for 10 min at 25 °C and normalized to the DMSO control. Jasp, LatA, and CytD were used at 3 μM each and blebbistatin at 100 μM. Data points are means ± S.E. from a representative experiment performed in triplicate.
signal was not significantly affected by actin inhibitors or blebbistatin as compared with the DMSO control (Fig. 4C). Collectively, our findings imply that actin and other components of the cytoskeleton play a role in HIV-1-mediated cell-cell fusion but are dispensable for virus-cell fusion.

**Actin Drives Late Steps of HIV-mediated Cell-Cell Fusion**—Actin has been implicated in different steps of HIV-1 Env-mediated cell-cell fusion. Several reports suggested that actin is involved in early steps of fusion, such as CD4/coreceptor clustering (26, 52–56). Similarly, terminal enlargement of fusion pores between cells leading to syncytia formation is modulated by actin (57, 58). To discern the potential role of actin in CD4/coreceptor-binding steps from its role in viral fusion, we captured HIV-1 fusion at a temperature-arrested stage referred to as TAS (12, 25, 59). This fusion intermediate is created upon prolonged incubation at a subthreshold temperature for fusion (18–24 °C, depending on the experimental system), which allows Env to engage CD4 and coreceptors but prevents downstream refolding of gp41 and membrane fusion (Fig. 5A) (12, 25). The formation of ternary Env-CD4-coreceptor complexes at TAS is manifested in partial resistance of fusion induced by raising the temperature to high doses of CD4 and coreceptor binding inhibitors that otherwise fully block fusion when present throughout the virus-cell incubation step (data not shown).

We next asked whether HIV-1 fusion remained sensitive to actin inhibitors added at TAS. To create TAS using the overlay system (Fig. 1D), cells and viruses were incubated for 3 h at 20 °C. The formation of Env-CD4 and Env-CD4-coreceptor
Deletion of the gp41 Cytoplasmic Tail Selectively Enhances FFWO—HIV-1 maturation and the gp41 cytoplasmic tail have been recently shown to control the formation of Env clusters on viral particles (60). Using super-resolution microscopy, the authors found that most mature particles contained a single wild type (WT) Env cluster, whereas the tail-deleted Env mutant (ΔCT) tended to form multiple clusters per particle. Importantly, unlike WT Env, multiple ΔCT Env clusters appeared to coalesce into a single cluster upon contact with CD4-expressing cells (60). These results show that CT restricts lateral movement of Env and suggest that the ability of Env to cluster is essential for their fusion activity.

Because viruses inducing fusion between adjacent cells should form at least two distinct fusion pores, we asked whether unrestricted lateral mobility of ΔCT Env can increase the likelihood of FFWO by forming functional clusters at the sites of virus contact with adjacent CD4-expressing cells. HIV-1 pseudoviruses carrying WT or ΔCT NL4-3 Env were produced and characterized by p24 ELISA (data not shown) and Western blotting (Fig. 6B). The usage of NL4-3 Env in these experiments is dictated by a more uniform incorporation of WT and ΔCT Env into virions and cleavage of the gp160 precursor (Fig. 6B) compared with HXB2 Env (data not shown).

Comparison of the fusion activity and infectivity of WT and ΔCT viruses was performed using equal amounts of p24 in viral inoculum. In agreement with the previous reports (33, 60, 61), viruses bearing ΔCT Env exhibited lower specific infectivity compared with WT (Fig. 6A). However, despite the lower specific infectivity, ΔCT viruses fused with cells almost twice as efficiently as WT (Fig. 6C). Compared with a relatively modest effect on virus-cell fusion, deletion of the gp41 CT dramatically (17-fold) promoted FFWO (Fig. 6D). The enhanced FFWO induced by ΔCT Env was sensitive to Jasp pretreatment (Fig. 6E), showing that efficient cell fusion induced by this mutant remained actin-dependent. Thus, the gp41 CT appears to selectively restrict the ability of pseudoviruses to fuse adjacent cells. These results suggest that the apparent ability of ΔCT Env to form clusters at the virus-cell contact sites (60) is critical for
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FFWO, which likely proceeds through formation of at least two distinct fusion pores.

HIV-1 Uptake and Fusion with CD4\(^+\) T Cells Are Partially Dependent on Intact Actin— Whereas HIV-1 fusion with HeLa-derived cells is not affected by actin disruption (13, 48, 62), the actin cytoskeleton has been implicated in infection of CD4\(^+\) T cells (53, 63). We therefore examined the actin dependence of HIV-1 fusion with the lymphoid cell line, CEM.NKR-CCR5-Luc, engineered to express CCR5 and containing a Tat-inducible luciferase expression cassette (31). Viruses were allowed to bind and fuse to these cells (hereafter referred to as CEM.CCR5 cells), as described under “Experimental Procedures.” Cells were then loaded with the CCF4-AM substrate and adhered to poly-lysine-coated 96-well plates prior to reading the BlaM signal. Virus-cell fusion was significantly diminished in the presence of actin inhibitors (Fig. 7A). Jasp inhibited fusion less effectively than LatA or CytD; an ~30% drop in the BlaM signal was observed at 3 \(\mu\text{M}\) of Jasp, whereas the same effect was achieved in the presence of 0.2 \(\mu\text{M}\) of the other two inhibitors. Thus, in contrast to the HIV-1 fusion with adherent cell lines (Fig. 3C), actin appears to play a role in HIV-1 fusion with CEM.CCR5 cells. However, LatA abrogated HXB2 pseudovirus-mediated fusion (FFWO) between CEM.CCR5 cells (Fig. 7B), similar to FFFWO between adherent cells (Fig. 3C).

Next, we examined the role of other cytoskeleton components on HIV-1 fusion with CEM.CCR5 cells. Blebbistatin diminished the fusion efficiency (\(p < 0.001\)), whereas the microtubule inhibitors, nocodazole and paclitaxel, slightly increased the BlaM signal (Fig. 7C, \(p > 0.26\)). These results indicate that actin and myosin II may play a role in HIV-1 fusion with CEM.CCR5 cells.

We also assessed the effect of actin inhibitors on the VSV G pseudovirus (VSVpp) fusion with CEM.CCR5 cells. Whereas the effects of cytoskeleton inhibitors on the VSVpp fusion were generally similar to those observed for HIV-1 pseudoviruses, a few differences were noted (Fig. 7, A, C and D). Nocodazole markedly promoted VSVpp fusion, whereas the effect of Jasp was bi-phasic with nearly 50% reduction in fusion efficiency at a low concentration and virtually no inhibition at 3 \(\mu\text{M}\) (Fig. 7D).

The actin dependence of HIV-1 fusion with CEM.CCR5 cells could be indicative of virus fusion with the plasma membrane, and has been recently suggested (24). However, the diminished VSVpp fusion with acidic endosomes (64) in the presence of actin inhibitors suggests that actin may be involved in productive virus uptake by these cells rather than the fusion step itself. To examine the role of actin in HIV-1 internalization, CEM.CCR5 cells were allowed to bind viruses in the cold and were washed and incubated for 2 h at 37 \(^\circ\text{C}\). Virions remaining at the cell surface were removed by trypsin, and the amount of intracellular p24 was measured by ELISA. Untreated cells and cells incubated with respective amounts of DMSO internalized nearly 30% of cell-bound HIV-1 pseudoviruses within 2 h, whereas cells pretreated with actin-disrupting drugs seques- tered the virus 2–3-fold less efficiently (Fig. 7E, \(p < 0.024\)). Thus, in contrast to the HIV-1 uptake by adherent cells (Fig. 3B), actin plays a role in virus endocytosis by CEM.CCR5 cells. The roughly 2-fold decrease in both HIV-1 fusion and bulk virus uptake in CEM.CCR5 cells pretreated with actin inhibi-
clathrin-dependent uptake of smaller (<100 nm) viral particles does not require actin remodeling (73), it is unlikely that HIV-1 particles experience considerable lateral forces prior to their internalization. Consistent with this notion, pharmacological disruption of actin filaments does not block HIV-1 fusion with adherent cells (see Refs, 13, 48, 62; see Ref. 46 for the opposite result). By comparison, actin rearrangement induced in target cells upon contact with Env-expressing cells (26, 47) or by HIV-1 particles sandwiched between target cells could augment the pore dilation through generating a lateral force within the PM.

The finding that Abl kinase inhibitors block HIV-1 Env-mediated fusion at a hemifusion stage (46) is consistent with the requirement for an external (actin-generated) force to initiate/
enlarge a fusion pore. Interestingly, myoblast fusion in the course of muscle development is also driven by actin (74), suggesting a universal link between actin polymerization and force generation required for cell fusion. However, studies of syncytia formation induced by viral fusion proteins, such as influenza HA, baculovirus gp64, and parainfluenza virus 5 F, imply that actin can restrict the growth of large micron-size pores between fusing cells (57, 58, 75). Thus, actin rearrangements could be rate-limiting for large scale changes in the morphology of fusing cells. These results do not appear to conflict with the notion that actin drives the dilation of small fusion pores to sizes (<100 nm) that allow the viral nucleocapsid release into the cytoplasm.

Role of gp41 Cytoplasmic Tail in HIV-1 Fusion—The dramatic increase in the efficiency of FFWO upon deletion of the gp41 CT is consistent with the notion that these fusion events are disfavored by constraints on the lateral mobility of Env imposed by interactions between the CT and the matrix protein lattice in mature particles (33, 76). We surmise that the lateral mobility of ΔCT Env could facilitate the formation of two spatially distinct fusion complexes on the same particle mediated by interactions with CD4 on two adjacent cells, a possibility that is supported by super-resolution imaging (60). In contrast, WT Env primarily resides in a single cluster on mature virions, which should disfavor the formation of two distinct functional fusion sites required to induce FFWO. However, a single WT Env cluster appears essential for productive entry/fusion of free viruses, as evidenced by the greater specific infectivity of WT compared with the ΔCT mutant (Fig. 6A) (60). Our results are consistent with the recent finding that, although a greater number of ΔCT Env can be incorporated into virions produced by certain cell types, these virions are less infectious than those bearing WT Env (61). At the same time, the enhanced fusogenic activity of the tail-deleted Env (77) is likely to augment HIV-cell fusion.

HIV-1 Entry into CD4+ T Cells—Diverse viruses rely on the cytoskeleton for their entry and replication (78–82). HIV-1 has been reported to hijack actin, although the exact mechanism by which actin aids the virus entry is not completely understood (53, 63, 78, 83). The effect of actin on HIV-1 entry appears to be cell type- and experimental condition-dependent, ranging from inhibition of infection by cortical actin in resting CD4+ T cells (63) to promoting CD4/coreceptor clustering and infection (53, 54, 84) and cell-to-cell transmission (85). HIV-1 entry into macrophages has been shown to be actin-dependent (14, 19, 20). Post-fusion steps of entry have also been reported to rely on actin (86).

Unlike the adherent NP2-derived (this study) and HeLa-derived cells (13, 48, 62), in which the HIV-1 fusion appears to occur in an actin-independent manner, disruption of actin filaments significantly reduced fusion with CEM.CCR5 cells. Our finding that actin inhibitors also reduce the efficiency of virus uptake by CEM.CCR5 cells is consistent with published data on the actin dependence of HIV-1 uptake by CD4+ T cells (83). Although these results do not rule out the possibility of HIV-1 fusion with the PM of lymphoid cells, as reported previously (24), the proportional decrease in the virus uptake and fusion in CEM.CCR5 cells pretreated with actin inhibitors is in line with fusion occurring after CD4/coreceptor-mediated and actin-dependent endocytosis. This notion is further supported by the requirement for intact actin for acid-mediated VSVpp fusion with CEM.CCR5 cells.

Possible in Vivo Relevance—An alternative explanation for the observed inhibition of HIV-1 fusion with CEM.CCR5 cells upon actin disruption could be the high cell and virus densities. These experiments involved co-pelleting of viruses with cells (see “Experimental Procedures”), which should increase the number of cell-cell contacts mediated by viruses and thus enable cells to apply a force to these particles. These conditions could be satisfied in vivo. Indeed, a high density of CD4+ T cells in lymph nodes of infected humanized mice appears to favor syncytia formation (87). In addition, HIV-1 particles transmitted through virological synapses could, at least temporarily, be attached to both cells across the contact area (88). The fact that cell-to-cell transmission does not normally lead to cell fusion suggests negative regulation of this entry pathway, perhaps through delayed virus maturation (89) or through host factors interfering with the fusion process (90, 91). If complete HIV-1 fusion at the cell surface relies on actin, what drives pore dilation in endosomes following CD4- and coreceptor-mediated uptake? It is possible that local forces applied to endosomal membranes undergoing fission and fusion during vesicular trafficking and sorting (92–94) create lateral tension and thereby dilate a pore created by viral proteins.

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Note Added in Proof—Fig. 6B was not formatted correctly in the version of this article that was published on January 14, 2015 as a Paper in Press. Fig. 6B has been revised to show that it was assembled from separate Western blot images.

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