

# Varying effects of temperature, $\text{Ca}^{2+}$ and cytochalasin on fusion activity mediated by human immunodeficiency virus type 1 and type 2 glycoproteins

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**Abstract** We examined fusion mediated by the human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) envelope glycoproteins under various experimental conditions. Incubation of HeLa cells expressing HIV-2<sub>ROD</sub> and HIV-2<sub>SBL/ISY</sub> envelope glycoproteins with HeLa-CD4 target cells resulted in fusion at temperatures  $\geq 25^\circ\text{C}$  whereas fusion with cells expressing HIV-1<sub>Lai</sub> occurred only at  $\geq 31^\circ\text{C}$ . HIV-2 envelope glycoprotein-mediated fusion proceeded in the absence of  $\text{Ca}^{2+}$  in the culture medium, whereas HIV-1 fusion required  $\text{Ca}^{2+}$  ions for fusion. In contrast to HIV-2 envelope glycoprotein fusion, incubations in the presence of the 0.5  $\mu\text{M}$  cytochalasin B completely inhibited HIV-1 envelope glycoprotein-mediated fusion. Our results suggest that in contrast to HIV-2, HIV-1 fusion is dependent on dynamic processes in the target membrane.

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**Key words:** Fusion activity of HIV-2 envelope glycoprotein

## 1. Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) enter permissive cells by interaction with the cellular receptor CD4, and an appropriate chemokine coreceptor followed by fusion of viral and target membranes [1]. Despite a similar genetic organization of HIV-1 and HIV-2, their amino acid sequences are considerably divergent (see the HIV sequence database on <http://hiv-web.lanl.gov/>). HIV-1 is primarily prevalent in the USA and Europe, whereas HIV-2 is endemic in West Africa as well as the west coast of India (see epidemiological data on <http://www.who.org/>). A number of distinct differences between HIV-1 and HIV-2 envelope glyco-

proteins in their ability to mediate viral entry have been reported: (a) a broad range of chemokine receptors are used by HIV-2 as coreceptors with CD4 [2,3], whereas HIV-1 is more restricted in its host range and utilization of chemokine receptors, (b) naturally occurring strains of HIV-2 envelope glycoprotein have been identified which promote efficient fusion and viral entry in the absence of target cell CD4 [4], whereas CD4-independent HIV-1 envelope glycoprotein phenotypes have only been produced in the laboratory [5], (c) in contrast to HIV-2, entry mediated by HIV-1 envelope glycoprotein requires presence of glycosphingolipids in the target membrane [6].

An examination of differences in fusion phenotype between two closely related viruses may lead to a better understanding of the molecular interactions of envelope proteins with the targets leading to fusion. Therefore, we studied the effects of temperature,  $\text{Ca}^{2+}$  and F-actin polymerization on fusion activity mediated by HIV-1 and HIV-2 envelope glycoproteins. We report here that the temperature threshold for HIV-2 envelope glycoprotein-mediated fusion is lower as compared to that for HIV-1. HIV-2 envelope glycoprotein-mediated fusion does not require the presence of  $\text{Ca}^{2+}$  in the incubation medium although fusion is reduced in the absence of  $\text{Ca}^{2+}$  ions. Moreover, HIV-2 envelope glycoprotein-mediated fusion is not affected by cytochalasin B, which impairs F-actin polymerization in the cytoskeleton. Thus we observe considerable differences in fusion phenotype between HIV-1 and HIV-2 envelope glycoproteins when physicochemical conditions are varied.

## 2. Materials and methods

Fluorescent probes were obtained from Molecular Probes (Eugene, OR, USA) and tissue culture media were from Gibco BRL (Life Tech, Gaithersburg, MD, USA). Other reagents were from Sigma Chemical Company (St. Louis, MO, USA).

### 2.1. Cells

HeLa cell lines stably expressing human CD4 (HeLa-CD4) and the parental HeLa cells were a gift from Dr. John Silver. NIH 3T3 cells stably expressing human CD4 (3T3-CD4) were a gift from Dr. Dan Litman (Howard Hughes Medical Institute). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (D10).

### 2.2. Expression of HIV envelope proteins

HIV envelope proteins were transiently expressed on the surface of HeLa cells using the following recombinant vaccinia constructs: (i) rVV/ROD (encoding the gene for HIV-2<sub>ROD</sub> envelope under the control of the vaccinia P7.5 early/late promoter); (ii) rVV/ST (encod-

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**Abbreviations:** HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; DMEM, Dulbecco's modified Eagle's medium; D10, DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin; PBS, phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4; EBSS, Earle's balanced salt solution; EBSS- $\text{Ca}^{2+}$ , EBSS without calcium and magnesium; EBSS+ $\text{Ca}^{2+}$ , EBSS supplemented with 1.8 mM calcium; CMTMR, (5-(and 6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine

ing HIV-2<sub>ST</sub> envelope gene under the control of the vaccinia P7.5 early/late promoter) [7]; (iii) vSC50 (expressing HIV-2<sub>SBL/ISY</sub> envelope, an infectious molecular clone of isolate SBL6669 under the control of the vaccinia P7.5 promoter) [8]; (iv) vPE16 (expressing HIV-1<sub>Lai</sub> (BH8) envelope). The infections were done as described [9]. Briefly,  $2 \times 10^6$  HeLa cells were plated on a 75 cm<sup>2</sup> tissue culture flask 6–8 h prior to infection. The virus (10 multiplicity of infection) was allowed to bind to the cells in 1.5 ml of DMEM containing 2% FBS for 2 h at 37°C. The cells were removed from the flasks with mild trypsinization and resuspended in D10. The infection was continued further for 14–16 h at 31°C, at which time the infected cells were washed and used in a fusion assay as described below.

### 2.3. HIV envelope glycoprotein-mediated cell–cell fusion

To monitor cell–cell fusion, we used HeLa-CD4 and 3T3-CD4 as target cells and gp120–gp41-expressing HeLa cells as effectors. The target cells were plated on 12-well tissue culture plates at  $10^5$  cells per well, and labeled with the cytoplasmic dye (5-(and 6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) (10 μM, ex/em 550/565 nm) [6]. The HIV envelope glycoprotein-expressing cells were labeled with the fluorescent cytosolic probe calcein AM (10 μM, ex/em 492/516 nm). Envelope-expressing cells ( $5 \times 10^4$ ) were added to the respective adherent fusion partners on the 12-well plates in 1 ml of medium. The two cell populations were incubated for 3 h at various temperatures. To determine the role of Ca<sup>2+</sup> ions, the effector and target cells were washed several times with the Dulbecco's phosphate-buffered saline (PBS) (without Ca<sup>2+</sup>/Mg<sup>2+</sup>) to remove calcium and then resuspended either in Earle's balanced salt solution without calcium (EBSS–Ca<sup>2+</sup>) or EBSS supplemented with 1.8 mM calcium (EBSS+Ca<sup>2+</sup>). Target and effector cells were co-cultured at 37°C as described above in EBSS–Ca<sup>2+</sup> or EBSS+Ca<sup>2+</sup>. In experiments to study the effect of cytoskeletal elements, incubations were done in the presence or absence of 0.5 μg/ml cytochalasin B [10]. At the end of incubations, the phase and fluorescence images were collected. Image analysis for dye mixing was performed with Metamorph image analysis software as described [6]. Bright field images were used to distinguish false positives where labeled cells were lying over one another but had not actually fused. Percent fusion is calculated as: % fusion =  $100 \times (\text{number of nuclei positive for both dyes}) / (\text{total number of nuclei})$ .

## 3. Results

### 3.1. Temperature dependence of cell–cell fusion induced by the HIV envelope glycoprotein

Previous studies have shown that HIV-1 envelope glycoprotein-mediated fusion with CD4<sup>+</sup> cells is a temperature-dependent process and fusion occurs only at  $\geq 31^\circ\text{C}$  [11]. To determine whether HIV-2-mediated fusion follows a similar temperature dependence, we studied fusion of two CD4-dependent X4-utilizing HIV-2 envelope glycoproteins (HIV-2<sub>ROD</sub> and HIV-2<sub>SBL/ISY</sub>). Cells expressing the HIV-2<sub>ST</sub> envelope glycoprotein which do not fuse with HeLa-CD4 cells [12] were used as controls. Fig. 1A shows images of calcein-loaded envelope-expressing cells following incubation with CMTMR-labeled HeLa-CD4 cells for 3 h at 25°C. Dye redistribution as a result of fusion occurred with HIV-2<sub>ROD</sub>- and HIV-2<sub>SBL/ISY</sub>-expressing cells, but not with the cells expressing the HIV-1<sub>Lai</sub> envelope glycoprotein. Fig. 1B shows data collected from many such images as a function of temperature. Cells expressing HIV-2<sub>SBL/ISY</sub> and HIV-2<sub>ROD</sub> glycoprotein fused with HeLa-CD4 target cells at the lower temperatures (25°C and 28°C) whereas no fusion with HIV-1<sub>BH8</sub>-expressing cells was observed. There was no fusion with the HIV-1 envelope glycoprotein-expressing cells even after incubations with target cells for 24 h at 25°C. No fusion was observed when cells expressing HIV-2<sub>ST</sub> strain were incubated with HeLa-CD4 cells at all the temperatures studied, indicating that the dye transfer resulted from specific fusion.

### 3.2. HIV-2 envelope glycoprotein-mediated fusion does not require Ca<sup>2+</sup>

Previous studies have shown that membrane fusion between HIV-1 gp120–gp41-expressing and CD4<sup>+</sup> cells requires calcium ions in the incubation medium [13]. To examine whether calcium ions are also required for HIV-2 envelope glycoprotein-induced fusion with CD4<sup>+</sup> cells, we co-cultured HeLa-CD4 and gp120–gp41 (from HIV-1 or HIV-2 envelope)-expressing cells at 37°C in the presence of incubation medium devoid of calcium ions (EBSS–Ca<sup>2+</sup>). Fusion was monitored by observing formation of syncytia as well as by transfer or fluorescent probes between fusing cells. Cells expressing HIV-1<sub>Lai</sub> and HIV-2 envelope (HIV-2<sub>ROD</sub> and HIV-2<sub>SBL/ISY</sub>) fused with HeLa-CD4 cells as judged by the formation of syncytia in the presence of EBSS+Ca<sup>2+</sup>, consistent with previous observations (Fig. 2A). On the other hand, when incubations were performed in EBSS–Ca<sup>2+</sup>, fusion was only observed with HIV-2 but not HIV-1 envelope-expressing cells (Fig. 2B). However, a reduction in the formation of syncytia was observed in EBSS–Ca<sup>2+</sup> as compared to the incubations done in EBSS+Ca<sup>2+</sup> (Fig. 2A). Using the dye redistribution assay, we show that cells expressing HIV-2 envelope glycoprotein fused with HeLa-CD4 cells when incubated in EBSS–Ca<sup>2+</sup>. Incubation of HeLa-CD4 and HIV-1 envelope-expressing cells in EBSS–Ca<sup>2+</sup>, however, did not show any dye transfer consistent with previously reported observations [13]. We did not observe any dye transfer when HIV-2 envelope glycoprotein-expressing cells were incubated with NIH 3T3-CD4 cells (CXCR4 negative) as targets (Fig. 2B), confirming specificity of dye transfer as a result of fusion. Although calcium ions are not required for HIV-2 fusion, fusion was enhanced in the presence of Ca<sup>2+</sup> ion in the culture medium (Fig. 2A,B).

### 3.3. Effects of cytoskeletal dynamics on HIV-2 envelope glycoprotein-mediated fusion

It has been shown previously that treatment of target cells with cytochalasin B [11] and D [14] blocks HIV-1 envelope glycoprotein-mediated fusion, presumably by inhibiting actin polymerization. Our observation that HIV-2 envelope glycoprotein-mediated cell–cell fusion occurs at lower temperatures (Fig. 1) and does not require calcium ions (Fig. 2) suggests that the cellular dynamics involved in HIV-2 envelope glycoprotein-mediated fusion might be different than that for HIV-1. We therefore tested the effect of cytochalasin B, which affects cytoskeletal dynamics on HIV-2 fusion. Fig. 3 shows that syncytia formation mediated by the HIV-1 envelope glycoprotein was completely inhibited in the presence of 0.5 μM cytochalasin B, in accordance with previous studies [11]. However, there was no inhibition of HIV-2-mediated fusion and syncytia formation under identical conditions. These observations were confirmed using the dye redistribution assay described in Fig. 1 (data not shown). Therefore, in contrast to HIV-1, cytoskeletal dynamics do not appear to play an important role in HIV-2 envelope glycoprotein-mediated fusion.

## 4. Discussion

HIV-1 and HIV-2 infect susceptible cells by fusion of the viral membrane with the cell plasma membrane. This process is mediated by the interactions of the envelope glycoproteins with CD4 and coreceptors on the host cell surface [9], that lead to the conformational changes enabling gp41 to form a

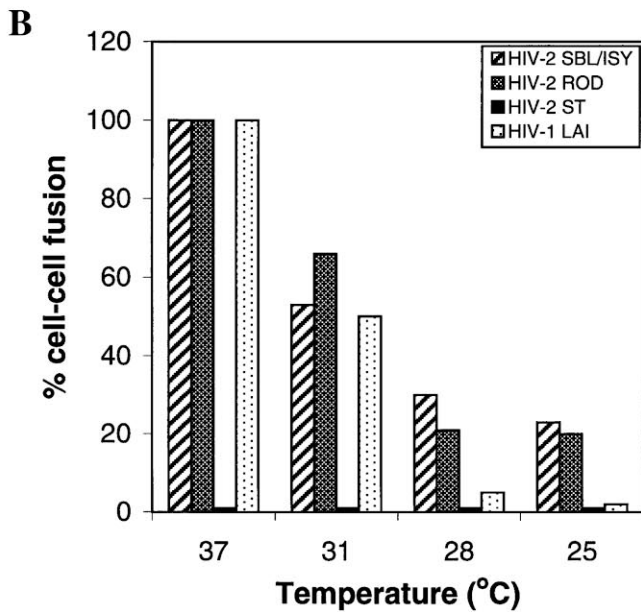
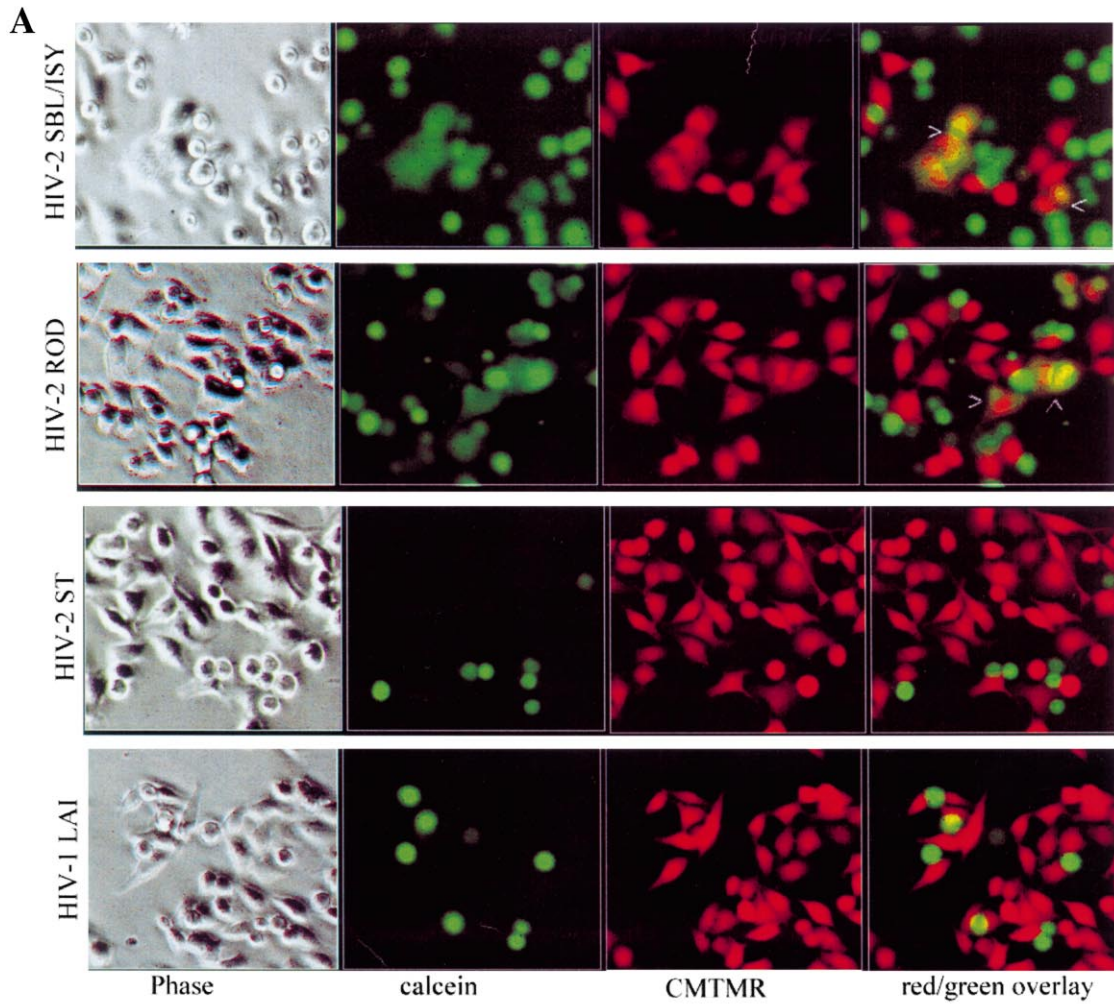


Fig. 1. Temperature dependence of HIV-1 and HIV-2 envelope glycoprotein-mediated fusion. Target cells were plated on 12-well tissue culture plates at  $10^5$  cells per well, and labeled with the cytoplasmic dye CMTMR (10  $\mu$ M, ex/em 550/565 nm). HeLa cells were infected with the vaccinia recombinants to express viral proteins on the cell surface and labeled with the fluorescent cytosolic probe calcein AM (10  $\mu$ M, ex/em 492/516 nm) and resuspended in D10.  $5 \times 10^4$  envelope-expressing cells were added to the wells containing HeLa-CD4 cells and incubated for 3 h at various temperatures in a humidified incubator and 5% CO<sub>2</sub>. At the end of incubations, images were collected using a 20 $\times$  objective and fusion was scored using the assay described in the text. A: A micrograph showing fusion at 25°C. Red-green overlays were done using MetaMorph software (Universal Imaging Inc.). Positive fusion is indicated by >. B: Extent of fusion at various temperatures.

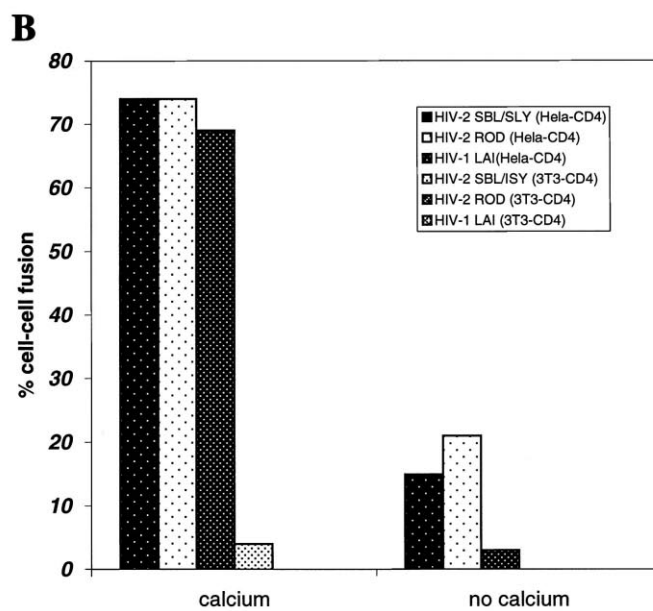
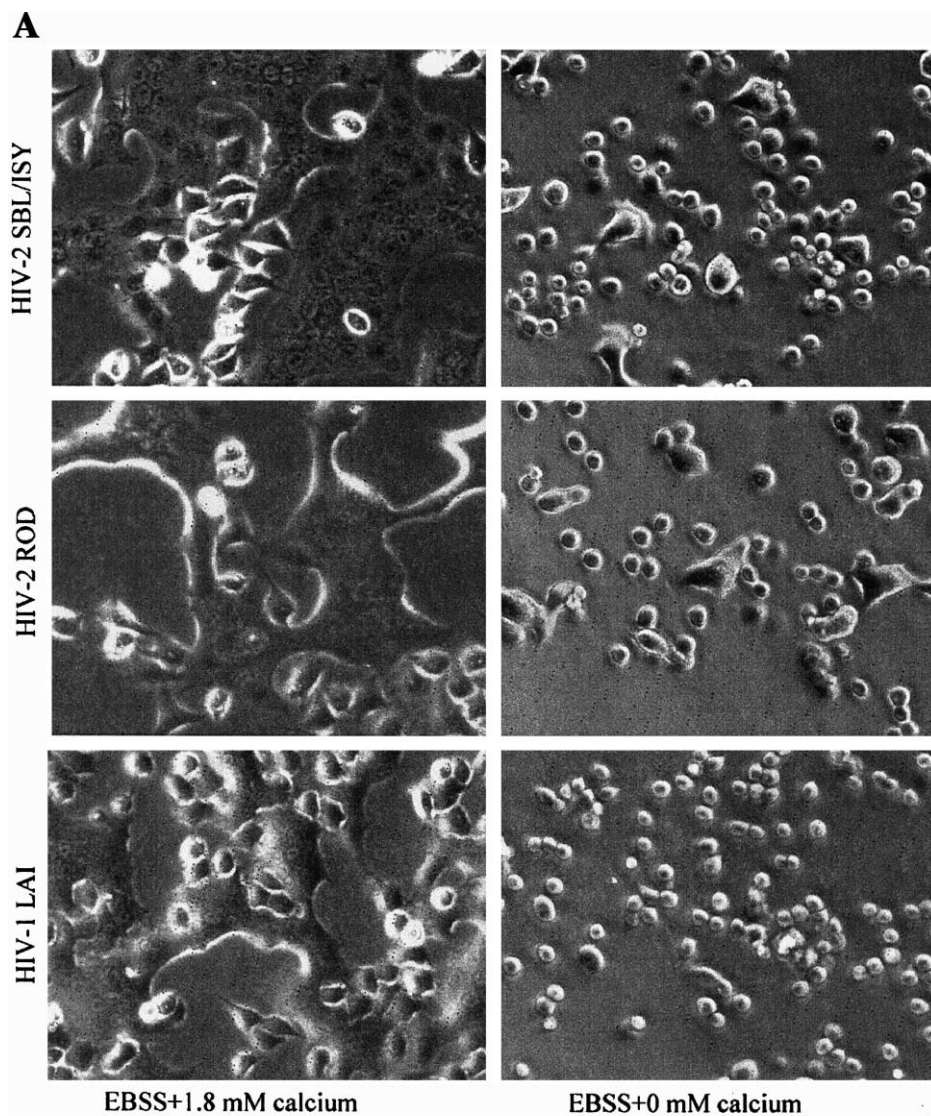




Fig. 2.  $\text{Ca}^{2+}$  dependence of HIV-1 and HIV-2 envelope glycoprotein-induced fusion. HeLa-CD4 cells plated on 12-well tissue culture plates were labeled with CMTMR and envelope-expressing cells were labeled with calcein described in the legend to Fig. 1. Cells were washed several times with Dulbecco's PBS (without  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ), and were either resuspended in EBSS+ $\text{Ca}^{2+}$  or EBSS- $\text{Ca}^{2+}$ . Target and effector cells were co-cultured for 3 h at 37°C (see legend to Fig. 1). At the end of incubations, images were collected using a 20× objective and fusion was scored following procedures described in the text. To distinguish small multinucleated cells occasionally found in cell cultures in the absence of fusion, formation of syncytia was confirmed by monitoring presence of CMTMR and calcein in the same population of cells (not shown). A: A micrograph showing fusion of HIV envelope-expressing cells with HeLa-CD4 cells in the absence or presence of  $\text{Ca}^{2+}$  ions. B: Extent of fusion in absence or presence of  $\text{Ca}^{2+}$  ions. NIH 3T3-CD4 cells were used as negative controls in this experiment.

viral 'hairpin' [15,16] followed by assembly of the gp41 hairpins into a fusion pore [17]. Since it has been shown that various animal enveloped viruses can fuse with targets which do not produce energy (e.g. liposomes, red blood cell ghosts, plasma membrane vesicles) [18,19], it appears that the viral envelope glycoprotein-mediated fusion process per se does not require input of metabolic energy from the target cell [20]. For those viral envelope glycoproteins whose fusogenic structures comprise coiled coils [21], the energy required to bend lipids

into shapes required for fusion is hypothesized to be derived from the formation of these coiled coils [22].

However, HIV-1 envelope glycoprotein-mediated fusion seems to bear the hallmarks of a requirement for target cell energy sources. One of those hallmarks is temperature dependence: in mammalian cells most cellular metabolic processes occur at  $\sim 37^\circ\text{C}$ . Moreover, the requirement for  $\text{Ca}^{2+}$  and the inhibition of HIV-1 fusion by cytochalasin are most likely due to cellular dynamics which involve assembly and disassembly

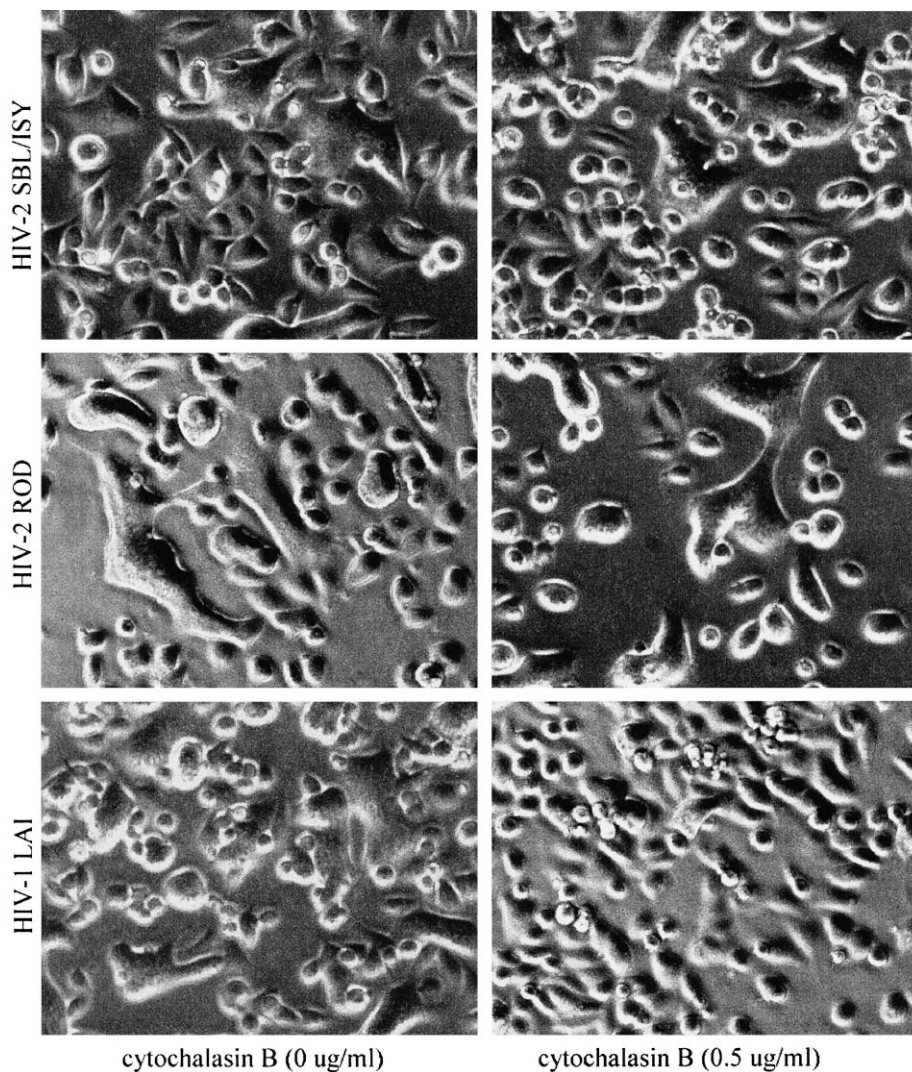


Fig. 3. The dependence of HIV-1 and HIV-2 envelope glycoprotein-mediated fusion on actin polymerization. HeLa-CD4 and HIV envelope-expressing cells were co-cultured (as described in the legend to Fig. 1) in the presence or absence of 0.5 µg/ml cytochalasin B for 3 h at 37°C. At the end of incubations, images were collected using a 20× objective. To distinguish small multinucleated cells occasionally found in cell cultures in the absence of fusion, formation of syncytia was confirmed by monitoring presence of CMTMR and calcein in the same population of cells (not shown).

of the actin network. It is unlikely that this energy is required to produce the conformational changes in HIV-1 gp120–gp41 since sCD4 induced shedding of gp120, which is an extreme manifestation of such conformational changes, occurs readily at 22°C with HIV-1 [23]. Moreover, in a study of varying temperature dependence of post-attachment neutralization of HIV-1 by monoclonal antibodies to gp120, changes in epitope exposure were demonstrated at temperatures of ~26°C [24].

Why then are these energetic factors important for HIV-1 envelope glycoprotein-mediated fusion? For HIV-1, it appears that massive conformational changes leading to aggregates of gp120–gp41–CD4 and coreceptor are involved in the initial stages of fusion [9,25]. It has recently been shown that addition of gp120 to activated peripheral blood mononuclear cells caused polarized co-capping of CD4 and CXCR4 with subsequent pseudopod formation [14]. Pretreatment of the cells with cytochalasin D blocked these membrane changes completely. Based on those observations, we hypothesize that confinement of gp120–gp41–CD4–coreceptor complexes in a limited area on the cell surface (co-capping) is essential to produce a local high concentration of transient gp41 pre-hairpins which then can self-assemble to form a fusion pore. If, in the case of HIV-1, the gp41 pre-hairpin formation is spread out over the cell surface, these molecules may inactivate before they self-assemble. This would occur when cellular dynamic processes are impaired at lower temperatures, absence of Ca<sup>2+</sup> or inhibition of actin polymerization. We surmise that in the case of HIV-2, the gp41 pre-hairpin is more stable and does not require the highly localized assembly of these structures. Therefore, the energy-dependent localization of the complexes (co-capping) is not required in the case of HIV-2 and fusion can proceed at lower temperatures, in the absence of Ca<sup>2+</sup>, and in the presence of cytochalasin B. The role of glycosphingolipids in the HIV-1 entry process [6,26,27] may also be related to the requirement for generation of gp41 pre-hairpins in localized areas on the cell surface. Further experiments are required to test this hypothesis.

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