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Physicochemical Dissociation of CD4-Mediated Syncytium Formation and Shedding of Human Immunodeficiency Virus Type 1 gp120

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The mechanism of CD4-mediated fusion via activated human immunodeficiency virus type 1 (HIV-1) gp41 and the biological significance of soluble CD4 (sCD4)-induced shedding of gp120 are poorly understood. The purpose of these investigations was to determine whether shedding of gp120 led to fusion activation or inactivation. BJAB cells (TF228.1.16) stably expressing HIV-1 envelope glycoproteins (the gp120-gp41 complex) were used to examine the effects of pH and temperature on sCD4-induced shedding of gp120 and on cell-to-cell fusion (syncytium formation) with $CD4^+$ SupT1 cells. sCD4-induced shedding of gp120 was maximal at pH 4.5 to 5.5 and did not occur at pH 8.5. At physiologic pH, sCD4-induced shedding of gp120 occurred at 22, 37, and 40°C but neither at 16 nor 4°C. In contrast, syncytia formed at pH 8.5 (maximally at pH 7.5) but not at pH 4.5 to 5.5. At pH 7.5, syncytia formed at 37 and 40°C but not at 22, 16, or 4°C. Preincubation of cocultures of TF228.1.16 and SupT1 cells at 4, 16, or 22°C before the shift to 37°C resulted in similar, increased, or decreased syncytium formation, respectively, compared with the control. Furthermore, an activated intermediate of CD4-gp120-gp41 ternary complex may form at 16°C; this intermediate rapidly executes fusion upon a shift to 37°C but readily decays upon a shift to the shedding-permissive but fusion-nonpermissive temperature of 22°C. These physicochemical data indicate that shedding of HIV-1 gp120 is not an integral step in the fusion cascade and that CD4 may inactivate the fusion complex in a process analogous to sCD4-induced shedding of gp120.

Binding of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120-gp41 complex via gp120 to its cell surface receptor, CD4, initiates a membrane fusion cascade (11, 36). Fusion of viral and plasma membranes is the major route for HIV-1 entry and consequently viral infection (35, 56). HIV-1-mediated syncytium formation is also dependent upon the interaction of CD4 and gp120-gp41 (28, 30, 31). The exact interaction of these membrane proteins leading to membrane fusion is incompletely understood. However, accumulated findings suggest that binding of CD4 to gp120 induces conformational changes in the noncovalently linked gp120-gp41 fusion complex (16, 37, 47), resulting in exposure and activation (1–4, 51) of the fusion domain in gp41 (8, 53). Activated gp41 probably effects membrane fusion by insertion of its hydrophobic amino terminus into the target cell membrane (18).

Soluble recombinant forms of CD4 (sCD4) mimic CD4 in binding to gp120 (12, 17, 22, 60) and are capable of inducing shedding of gp120 from virus and virus-infected cells (19, 27, 43). It has been suggested that sCD4-induced shedding of gp120 results in premature loss of fusogenic sites and contributes to inhibition of virus infection and syncytium formation (14, 42). However, on the basis of the notion of receptor-mediated activation of fusion (1–3), it has been suggested that shedding of gp120 may be an integral step in exposing the cryptic sites of gp41, thus priming for the fusion events (19, 43). Dissociation of sCD4-induced shedding of gp120 and fusion activation has been addressed by using site-directed mutagenesis of gp120 (7, 58). However, in these studies, the lack of correlation between shedding and fusion may simply represent the fact that a particular strain's or variant's envelope glycoprotein is either resistant to sCD4-induced shedding of gp120 or incapable of exerting fusion activation. Thus far, the biological significance of sCD4-induced shedding of gp120 is not clearly understood, nor is it known whether the events mediated by sCD4 reflect the interaction of CD4 and the gp120-gp41 fusion complex.

Physicochemical analyses have been widely used to dissect conformational changes of viral envelope glycoproteins which lead to membrane fusion. Mild acidic exposure (pH 5 to 6) is critical for entry of some viruses, such as Semliki Forest virus (21, 61) and influenza virus (52, 55). An acidic pH-independent process of the external membrane fusion has been described for HIV-1 as the major route of viral entry (35, 48, 56). However, the effects of pH on shedding of gp120 and syncytium formation have not been studied. Temperature affects the interaction of CD4 and gp120 (14, 40, 41, 59). Both shedding of gp120 (19, 47) and membrane fusion (15, 26, 48) are temperature sensitive. Our approach of studying the correspondence between shedding of gp120 and membrane fusion was to analyze the effects of pH and temperature on these events in a unique system. A BJAB-derived HIV-1 envelope glycoprotein-expressing cell line, TF228.1.16 (24), was used to study both sCD4-induced shedding of gp120 and CD4-mediated syncytium formation. This report demonstrates that both shedding of gp120 and syncytium formation are sensitive to pH and temperature and that the optimal conditions for each are different. The results of physicochemical analyses strongly suggest that CD4 and HIV-1 envelope glycoprotein-mediated membrane fusion are independent of receptor-induced shedding of gp120.

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MATERIALS AND METHODS

Cell lines. The HIV-1 envelope glycoprotein-expressing cell line TF228.1.16 (24) was derived by transfecting BJAB cells with a vector containing the full-length BH10 HIV-1 gp160 gene. The selected clone stably expresses functionally active gp120-gp41 on its surface. As do virus-infected cells, TF228.1.16 cells spontaneously release low levels of gp120 into the media. TF228.1.16 and SupT1 cultures were maintained in HY medium (GIBCO, Grand Island, N.Y.) supplemented with 15% fetal calf serum and antibiotics.

Analysis of shedding of gp120. TF228.1.16 cells were washed twice with HY medium to remove spontaneously released gp120 and resuspended at 5×10^7 cells per ml in culture medium supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; GIBCO). To induce shedding of gp120, sCD4 (SmithKline Beecham Pharmaceuticals) (12) was added to cell suspensions of various pHs in microcentrifuge tubes incubated in water baths at various temperatures. To quantify the spontaneous release of gp120 under various conditions, control cells were incubated with medium only. Cell-free supernatants were prepared by centrifugation and mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and the proteins were separated by electrophoresis (7.5% separating gel). Proteins were transferred to Immobilon membranes (Millipore, New Bedford, Mass.), and the membranes were probed with a rabbit antiserum against gp120 (kindly provided by R. Sweet, SmithKline Beecham Pharmaceuticals), detected with a horseradish peroxidase-linked donkey anti-rabbit antibody (Jackson ImmunoResearch, West Chester, Pa.), and visualized with the ECL detection system (Amersham). The optical density of protein bands was quantified with a densitometer (LKB), and the area under the density curve was measured with SigmaScan (Jandel Scientific, Corte Madera, Calif.) as described previously (19).

Analysis of syncytium formation. Suspensions of TF228.1.16 and SupT1 cells (5×10^6 cells per ml in HEPES-buffered culture medium) were mixed in microcentrifuge tubes. Cocultures were incubated statically to allow settlement of the cells at unit gravity at various pHs and temperatures. At the end of cocultivation, aliquots were diluted (1:10) with fresh HEPES-buffered culture medium or fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and then plated in triplicate in a 48-well plate. Giant cells (≥4 nuclei per cell) were counted with the aid of an inverted microscope while scanning through 1/10 of each well area. There were no differences observed in the syncytium counts obtained from live and fixed cells. This analysis of syncytium formation was validated by the methods of differential cytoplasmic fluorescent staining and flow cytometry.

Analyses of viability and metabolic activity. Trypan blue staining was used to examine cell viability. Cellular metabolic activity was analyzed by the tetrazolium-formazan assay (49), in which soluble colored formazan products were derived from metabolic reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT; Polysciences, Inc., Warrington, Pa.) and quantified by spectrophotometry.

RESULTS

Parameters of shedding of gp120 and syncytium formation. sCD4 induced a dose-dependent shedding of gp120 into the culture medium (Fig. 1A). Half-maximal shedding of gp120 was obtained with sCD4 at 50 μg/ml. Similar concentrations

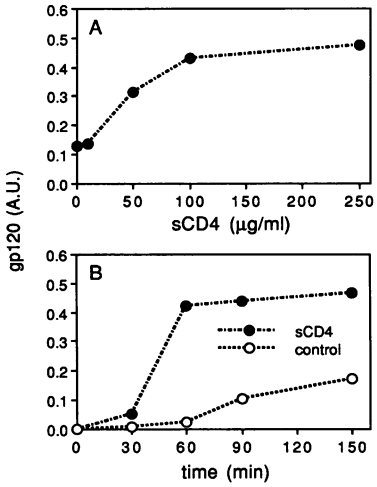

FIG. 1. sCD4-induced shedding of gp120 from TF228.1.16 cells. (A) TF228.1.16 cells were incubated in the absence or presence of sCD4 for 90 min at 37°C in physiologic pH culture medium. gp120 shed into the culture supernatant was identified by Western immunoblotting. The intensity of the gp120 band was analyzed by densitometry, and the area under the optical density curve was measured and expressed in arbitrary units (A.U.). (B) Time course of spontaneous and sCD4-induced shedding of gp120 from TF228.1.16 cells in the absence or presence of sCD4 (100 μg/ml). sCD4-induced shedding of gp120 was slow during the initial 30 min and was followed by rapid shedding until 60 min, after which time gp120 accumulated in the culture supernatant at a much slower rate. A slow, steady spontaneous release of gp120 was observed, with amounts ≤10% and ≤25% of those detected in the presence of sCD4 in 60- and 90-min incubation periods, respectively.

were required to induce shedding of gp120 from transfected cells expressing HIV-1 envelope glycoproteins (6, 43). This concentration is approximately 10-fold higher than that needed to induce shedding of gp120 from HIV-1 virions or infected cells (19, 27, 43). gp160, the precursor of the HIV-1 envelope glycoproteins, could be detected only in the cell pellets, indicating that gp120 release was not due to cell lysis. Cell pellet extracts derived from the cells treated with excessive sCD4 showed similar amounts of gp160 and significantly less gp120 than did controls.

The kinetics of sCD4-induced shedding of gp120 from TF228.1.16 was triphasic (Fig. 1B). The initial rate of sCD4-induced shedding of gp120 was slow for the first 30 min, progressed at a rapid rate between 30 and 60 min, and plateaued between 60 and 120 min. The initial 30-min lag period may represent a set of initial events of sCD4-gp120-gp41 interaction, including binding and conformational changes in the ternary complex of sCD4-gp120-gp41. In the absence of sCD4, gp120 was spontaneously released at a slow, steady rate that did not exceed 10% of that observed in the presence of sCD4 for 60 min. The kinetics of shedding of gp120 was similar to, although somewhat delayed in comparison with, that of HIV-1 virions or infected cells (6, 19, 27, 43).

The ability of TF228.1.16 cells to form syncytia with SupT1 cells was analyzed by counting the number of giant cells formed in cocultures (Fig. 2A). A syncytium was scored if it contained at least four nuclei. The time course of syncytium formation (Fig. 2B) exhibits a 30-min lag period similar to that observed for sCD4-induced shedding of gp120. However, this lag is probably due to the size criteria for scoring a syncytium (≥4 nuclei per cell), which would not

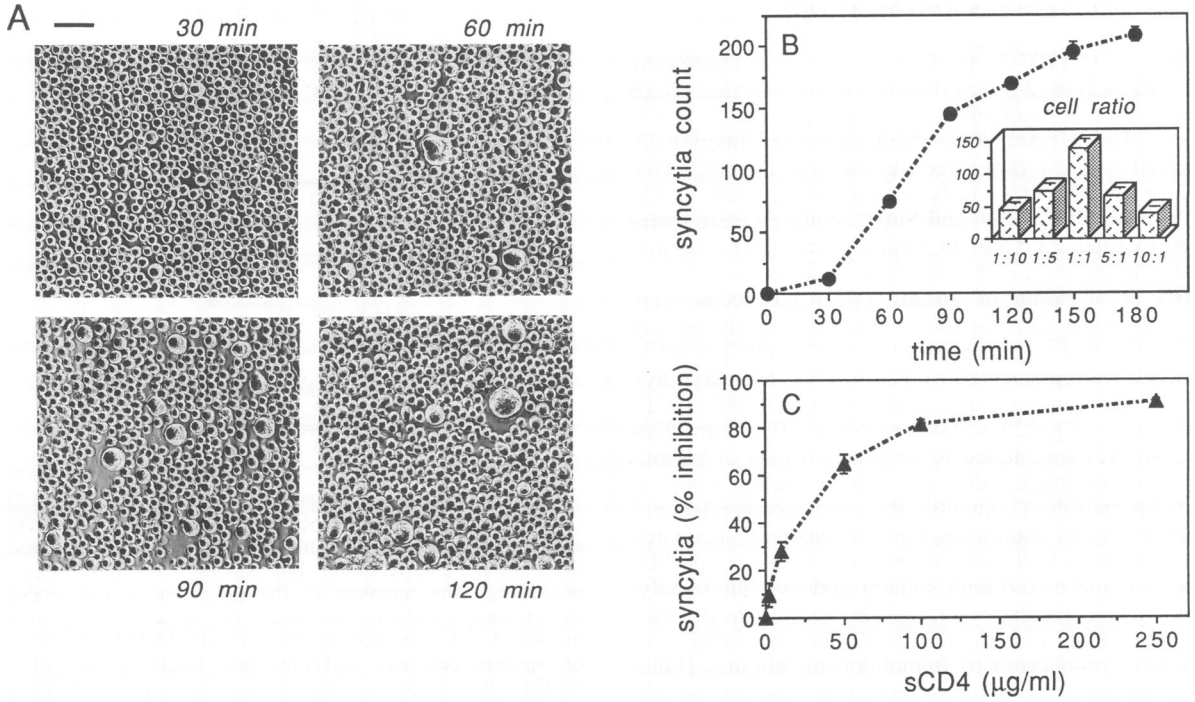

FIG. 2. Time course of syncytium formation and sCD4 concentration-dependent inhibition of syncytium formation. (A) Light micrographs of cocultures of TF228.1.16 and SupT1 cells at the indicated times. The bar equals 100 μm. (B) Syncytium counts. Syncytia (ca. ≥4 nuclei per cell) were scored from sequential aliquots taken following incubation at 37°C in pH 7.5 culture medium. A maximal number of syncytia formed in 90 min of incubation was seen at a 1:1 cell ratio (inset). The slow appearance of syncytia during the initial 30 min was due to the size limitation for syncytium scoring rather than to the immediate fusion events. A rapid increase in the number of syncytia was observed between 30 and 90 min. After 90 min, there was little increase seen in the absolute number of syncytia; however, the syncytia continued to grow in size (A). (C) sCD4 concentration dependence of blocking of syncytium formation. TF228.1.16 and SupT1 cells were incubated in the absence or presence of sCD4. Syncytium counts were obtained after 90 min of incubation in pH 7.5 medium at 37°C. Percent inhibition was calculated on the basis of the mean of syncytium counts of cocultures without sCD4 (0% inhibition), which gave a nominal range in syncytium number of 100 to 150 in repeated experiments. Half-maximal inhibition of syncytium formation was seen with approximately 25 μg of sCD4 per ml. Syncytium numbers are expressed as mean ± standard error of the mean (n = 3) for these experiments and those shown in Fig. 3 to 6.

detect initial cell-to-cell fusion events. After 30 min, we observed a rapid increase in the number of syncytia, which progressed until 90 min. Within this time frame of coculti-vation, the syncytia formed were generally consistent in size. The absolute number of syncytia increased slowly after 90 min, with the established syncytia continuing to recruit cells and increase in size. The effect of cell ratio (TF228.1.16/SupT1) on syncytium formation was investigated to deter-mine the optimal conditions. A symmetrical bell-shaped distribution plot of syncytium formation versus cell ratio with the peak centered at a 1:1 cell ratio was observed (Fig. 2B, inset). All further syncytium formation analyses were done with this cell ratio.

The capability of sCD4 to block syncytium formation in our assay system was evaluated to address the specificity of CD4-gp120-gp41 interaction-dependent syncytium formation for TF228.1.16 cells. Cocultures of TF228.1.16 and SupT1 cells were incubated in the presence or absence of sCD4 for 90 min, and syncytia were counted (Fig. 2C). Percent inhibition of syncytium formation was calculated on the basis of the syncytium count from cocultures without sCD4. Half-maximal inhibition of syncytium formation was ob-tained with sCD4 at 25 μg/ml.

sCD4-induced shedding of gp120 and syncytium formation are pH sensitive. The effects of pH on shedding of gp120 and syncytium formation were investigated in the established assay system for TF228.1.16 cells. In comparison with the standard levels obtained at pH 7.5, acidic media (pH 4.5 to 6.5) facilitated sCD4-induced shedding of gp120 from TF228.1.16 cells (Fig. 3A). In contrast, sCD4-induced shed-ding of gp120 at pH 8.5 was reduced to the background level. Spontaneous release of gp120 from TF228.1.16 cells was not affected by pH. The levels of spontaneous gp120 release were ≤20%, or equal to the sCD4-induced shedding of gp120 at pH 4.5 to 7.5 or at pH 8.5. The lack of sCD4-induced shedding of gp120 at pH 8.5 was not due to inhibition of sCD4 binding, since blockade of syncytium formation with sCD4 was observed at this pH.

Syncytium formation was maximal at pH 7.5 (Fig. 3B). Acidic medium (pH 4.5 to 5.5), which was optimal for sCD4-induced shedding of gp120, was unfavorable for syn-cytium formation. Although there was no sCD4-induced shedding of gp120 at pH 8.5, this basic pH reduced syncy-tium counts only mildly (ca. 25 to 30%).

The pH range investigated had little effect on cell viability or metabolic activity. Cells remained generally intact at pH 4.5 throughout the incubation period (90 min), as there was no detectable gp160 in the supernatants. In addition, more than 85% of the cells remain viable at pH 4.5 or 8.5, as evaluated by trypan blue exclusion. Similarly, when cell metabolic activity was assessed by tetrazolium-formazan assay, approximately 85% of the activity of the cells at pH 7.5 was seen for those incubated for 90 min at either pH 5.5 or 8.5. Neither the molecular integrity of gp120 nor its

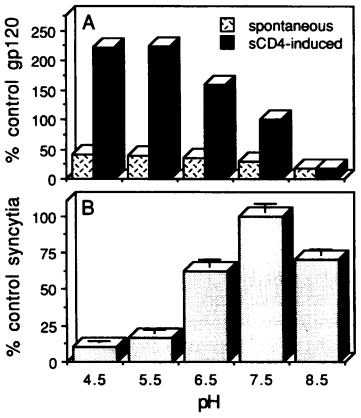

FIG. 3. Effects of pH on shedding of gp120 and syncytium formation. (A) TF228.1.16 cells were incubated in the absence or presence of sCD4 (50 μg/ml) for 90 min in media with a pH range of 4.5 to 8.5. A greater than twofold increase of sCD4-induced shedding of gp120 was detected in mildly acidic medium (pH 4.5 or 5.5) in comparison with that at pH 7.5 (control). sCD4 did not induce shedding of gp120 at pH 8.5. Spontaneous release of gp120 was not affected over the pH range tested. (B) The maximal number of syncytia formed at 37°C in 90 min was seen in the cocultures incubated at pH 7.5 (control) and hence designated 100%. The optimal pH for sCD4-induced shedding of gp120 gave negligible syncytium counts. Mildly reduced (ca. 25 to 30%) syncytium formation was seen at pH 8.5.

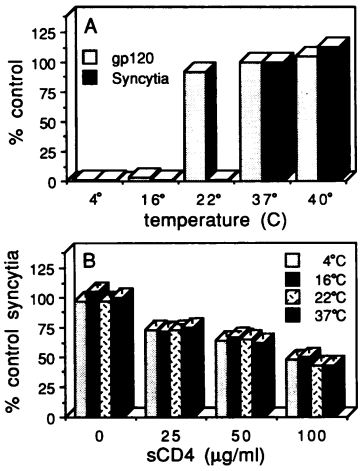

FIG. 4. Effects of temperature on sCD4-induced shedding of gp120 and syncytium formation. (A) TF228.1.16 cells were incubated with sCD4 (100 μg/ml) at 4, 16, 22, 37, or 40°C in pH 7.5 medium for 90 min. The levels of gp120 were standardized on the basis of that released at 37°C (control), which was designated 100% for quantitative comparison. Induced shedding of gp120 did not occur at either 4 or 16°C, but levels similar to the control levels were obtained at 22 and 40°C. In comparison, syncytium formation of TF228.1.16 and SupT1 cocultures was evaluated over the same range of temperatures incubated for 90 min. No syncytium formation was seen at and below 22°C. (B) TF228.1.16 cells were incubated in the absence or presence of sCD4 at various temperatures for 30 min, washed twice, and then cocultured with SupT1 cells for 90 min without exogenous sCD4. Control syncytium counts were derived from the cocultures incubated for 90 min without sCD4. Inhibition of syncytium formation was dependent on sCD4 concentration and independent of temperature during the preincubation with sCD4.

binding to CD4 was impaired by the acidic pH, as indicated by the levels of the spontaneous or sCD4-induced release of gp120 within the same course of incubation (90 min at 37°C) at low pH.

sCD4-induced shedding of gp120 and syncytium formation are temperature sensitive. It was demonstrated previously in our laboratory that sCD4-induced shedding of gp120 from HIV-1-infected cells does not occur at 4°C (19). By using the HIV-1 envelope glycoprotein-expressing cell line, this temperature dependence of sCD4-induced shedding of gp120 and syncytium-forming activity were studied in greater detail. sCD4-induced shedding of gp120 did not occur at either 4 or 16°C (Fig. 4A). However, there was no difference in the level of sCD4-induced shedding of gp120 observed following incubation at 22, 37, and 40°C. Little spontaneous release of gp120 was detected at all temperatures tested (control bar at pH 7.5 in Fig. 3A). In contrast, syncytium formation was not observed at temperatures of ≤22°C.

The absence of sCD4-induced shedding of gp120 at ≤16°C and of syncytium formation at ≤22°C was not due to a lack of binding of sCD4 or CD4 to gp120, as assessed in the following sCD4-mediated syncytium blocking experiment. TF228.1.16 cells were incubated in the presence and absence of sCD4 at 4, 16, 22, or 37°C for 30 min, washed twice to remove unbound sCD4, and then cocultured with SupT1 cells for 90 min at 37°C. As shown in Fig. 4B, inhibition of syncytium formation displayed an sCD4 concentration dependency despite different preincubation temperatures during the binding phase, indicating that equivalent binding of sCD4 or CD4 to gp120 of TF228.1.16 cells could occur at these temperatures.

Effect of preincubation temperature on syncytium formation. The differential temperature sensitivity of TF228.1.16 cells for shedding gp120 and forming syncytia allowed us to test the hypothesis that CD4 can act similarly to sCD4 in blocking syncytium formation, possibly via inducing shedding of gp120. Cocultures of TF228.1.16 and SupT1 cells were incubated at the fusion-nonpermissive temperature of 4, 16, or 22°C for 3 h and then shifted to 37°C for 90 min to allow syncytium formation. The syncytium counts were compared with those of the control, which was coculture incubated at 37°C for 90 min. The control syncytium counts were in the range of 100 to 125 in these and subsequent experiments. Compared with the control, similar, increased, or decreased syncytium formation was observed when the cocultures preincubated at 4, 16, or 22°C, respectively, were shifted to 37°C (Fig. 5A). Furthermore, separate cultivation of TF228.1.16 and SupT1 cells at these preincubation temperatures before the cells were mixed and shifted to 37°C resulted in syncytium formation similar to that of the control.

To evaluate the kinetics of the observed increase or decrease in the rate of syncytium formation seen at 16 or 22°C, respectively, a series of temperature shift experiments was conducted. Cocultures were incubated at 22°C (Fig. 5B) or 16°C (Fig. 5C) for variable intervals before the shift to 37°C for 90 min. Syncytium counts were compared with those of the control as described above. Preincubation of cocultures at 22°C for 30 min did not affect syncytium formation. However, after preincubation for 60 min, a significant decrease in syncytium formation at 37°C was observed (Fig. 5B).

In a similar set of experiments to examine the increased rate of syncytium formation, preincubation of cocultures at 16°C resulted in an opposite time course of temperature effect on syncytium formation (Fig. 5C). This priming effect

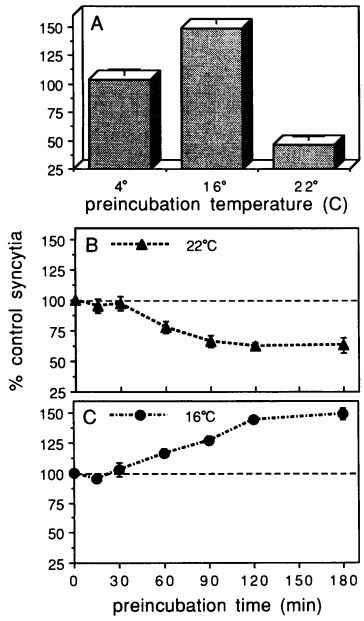

FIG. 5. Effects of preincubation temperature on syncytium formation. (A) TF228.1.16 and SupT1 cells were cocultured at 4, 16, or 22°C for 3 h and then shifted to 37°C for 90 min. Syncytium counts were compared and expressed as percent of the value for controls, which were incubated at 37°C for 90 min. Preincubation at 4°C did not affect syncytium formation. Compared with controls, cocultures preincubated at 16 or 22°C had a 50% increase or decrease, respectively, in syncytium formation. (B and C) The time course of the effects of preincubation temperature on syncytium formation was analyzed by coculturing the cells at 22°C (B) or 16°C (C) for various intervals before shifting them to 37°C for 90 min. Syncytium formation in these cocultures was compared with the control level as previously described. Syncytium formation was not affected by preincubation at either temperature ≤30 min. A decrease in syncytium formation was observed when the coculture was first incubated at 22°C for 60 min (B). An increase in syncytium formation was observed after 60 min and plateaued at 120 min following incubation at 16°C before the shift to 37°C (C).

plateaued after 120 min of preincubation at 16°C. At the plateau of priming, both the number and the size of syncytia formed at 37°C in 90 min were similar to those seen in the control cocultures incubated for ≥3 h (Fig. 2). Thus, this priming effect does not reflect an increase in the absolute number of fusion events but rather reflects an increase in the rate of fusion events.

Decay of the intermediate of fusion activation. Preincubation of cocultures at the shedding-permissive but fusion-nonpermissive temperature of 22°C decreased the rate of syncytium formation (Fig. 5A and B). These results suggest that fusion is abolished in a process that is probably analogous to sCD4-induced shedding of gp120. We next examined whether the priming for an enhanced rate of syncytium formation established during cocultivation at 16°C could be reversed by incubation at 22°C. Cocultures were incubated at 4, 16, or 22°C for 3 h, shifted to 22°C for variable intervals, and finally shifted to 37°C for 90 min (Fig. 6). Shifting of the cocultures preincubated at 16°C to the shedding-permissive temperature of 22°C before incubation at the fusion-permissive temperature of 37°C decreased the syncytium formation observed in a time (at 22°C)-dependent fashion. Incubation at 4°C for 3 h (0 min at 22°C) did not affect syncytium

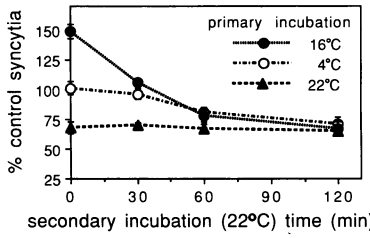

FIG. 6. Decay of the intermediate of fusion activation. Cocultures were first incubated at 4, 16, or 22°C for 3 h, shifted to 22°C for various intervals (x axis), and then shifted to 37°C for 90 min. Controls were cocultures incubated at 37°C for 90 min. The time course of inactivation of fusion was dependent on the previous phase of incubation temperatures. A faster reduction in syncytium formation was seen for cocultures following an initial incubation at 16°C than for those preincubated at 4°C. No further decrease in syncytium formation was seen upon further extension of the incubation time (≥3 h) at 22°C.

formation of the cocultures; however, when these cocultures were shifted to 22°C for increasing times, a decrease in syncytium formation at 37°C similar to that seen in Fig. 5B was observed. Following an initial incubation at 16°C, a significant reduction of syncytium formation was seen after the cocultures had been shifted to 22°C for ≥30 min. The decrease of syncytium formation by 22°C treatment proceeded at a faster rate for the cocultures preincubated at 16°C than for those preincubated at 4°C. No further decrease in syncytium formation was seen by further extending the incubation time (≥3 h) at 22°C.

DISCUSSION

This report describes a series of experiments in which we have studied the effects of pH and temperature on sCD4-induced shedding of HIV-1 gp120 and CD4-mediated syncytium formation. These experiments showed that both shedding of gp120 and syncytium formation were pH and temperature dependent and that there were marked differences in the pH optima and permissive temperatures for these phenomena. We have also found evidence that CD4 may inactivate HIV-1 envelope glycoprotein-mediated fusion in a similar fashion to that of sCD4. Furthermore, these data suggest that binding of CD4 to gp120 can lead to metastable activation of the fusion complex. These findings

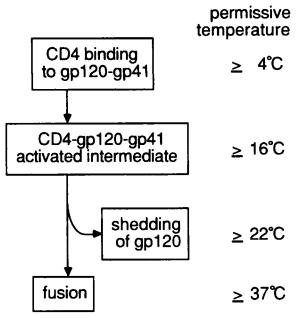

FIG. 7. Model of the stepwise fusion process, with the permissive temperatures listed for each step. An intermediate of fusion activation may follow receptor binding-initiated conformational changes. Shedding of gp120 is not an integral step in the fusion process and leads only to fusion inactivation.

allow us to propose a model shown in Fig. 7 for some of the events which occur between binding of CD4 to gp120 and cell-to-cell fusion.

For our studies, we used a recombinant human cell line stably expressing the transfected gene for full-length HIV-1 gp160. This system has allowed us to study the interaction of CD4 and gp120-gp41 without the potential complications introduced by other viral proteins. Because this system also provides rapid, high-efficiency syncytium formation, it allowed us to study the effect of pH and temperature on syncytium formation over a few hours rather than overnight, minimizing the impact of metabolic influences (protein synthesis, cell viability, etc.). Although it has been suggested that membrane fusion and syncytium formation are kinetically separable events (13), the rapid syncytium formation seen in our system minimizes the discrepancies that might exist between the two events.

Previous studies of the interaction of CD4 or sCD4 with gp120-gp41 have shown high-affinity binding (28, 29, 54) and sCD4-induced shedding of gp120 (6, 7, 14, 15, 19, 27, 41, 43). It has also been suggested that CD4 can induce shedding of gp120 (34). However, the biological significance of shedding of gp120 in relation to fusion activation is still incompletely understood. Antibodies against gp120 (38) or CD4 (44, 46) have been used to dissociate the event of CD4 binding to gp120 from that of fusion. These findings indicate that binding does not ensure fusion and that postbinding events are still susceptible to fusion blockade by specific antibodies. Studies using a mutagenesis approach to dissociate fusion from shedding of gp120 have shown that mutations within the V3 loop of gp120 block fusion but not sCD4-induced shedding of gp120 (7). These results indicate that shedding of gp120 does not ensure activation of fusion but do not rule out the possibility that shedding of gp120 is integral in fusion. Another study addressed the correlation between shedding of gp120 and fusion by examining a set of HIV-1 envelope variants which showed resistance to sCD4-induced shedding of gp120 but retained their fusion capability (58, 59). As we have shown in the present study, the physicochemical conditions (acidic pH or 22°C) which facilitated sCD4-induced shedding of gp120 were unfavorable for syncytium formation, and syncytium formation could occur at conditions (pH 8.5) in which sCD4 would not induce shedding of gp120. Assuming that the interaction of CD4 with gp120-gp41 is similar to that of sCD4, our data further support the notion that fusion is independent of shedding of gp120.

Receptor-mediated activation of fusion (2) has been described for several enveloped viruses. In addition, many enveloped viruses require physicochemical activation for fusion to occur. The results with immunodeficiency viruses are rather complicated. It has been suggested that CD4 activates HIV-1 envelope glycoprotein-mediated fusion (20), but sCD4 and sCD4-derived molecules have been shown to either inhibit (12, 17, 19, 22, 43, 51, 60) or enhance (3, 4, 9, 51, 63) viral infectivity or membrane fusion activity in different systems. These studies have shown marked differences among HIV-1, HIV-2, simian immunodeficiency virus, and their variant strains for receptor-mediated activation or inactivation of fusion, indicating that after receptor binding, the interaction between gp120 and gp41 is resolved differently in different systems. In addition to CD4, other molecules such as leukocyte adhesion factor LFA-1, which is required for HIV-mediated cell-to-cell fusion (45), may play a role in the process of fusion activation; however, they must act in context with the CD4-gp120-gp41 interaction.

One of the more intriguing results of our studies was the enhanced rate of syncytium formation observed when cocultures of TF228.1.16 and SupT1 cells were preincubated at 16°C and then shifted to 37°C (Fig. 5A and C). Preincubation of the cocultures at 4°C did not enhance the rate of syncytium formation. Our data on the ability of sCD4 to irreversibly block syncytium formation when preincubated at 4 or 16°C (Fig. 4B) suggest that these effects were not at the levels of CD4 binding to gp120.

Data from our temperature shift experiments also suggest that the intermediate of fusion activation established at 16°C may be metastable. As shown in Fig. 5B and C, there was an approximately 30-min lag for priming for both an increase (at 16°C) and a decrease (at 22°C) in syncytium formation. However, as shown in Fig. 6, when the cocultures were shifted from 16 to 22°C, there was an immediate and rapid decrease in syncytium formation, suggesting that the primed fusion activation is an intermediate step and can decay rapidly. Similar metastability of activated fusion proteins has been described for influenza virus (55) and Semliki Forest virus (61).

The results of our studies suggest that a variety of functionally significant events occur after CD4 binding to gp120-gp41. The precise mechanism of these events is unknown but may be a consequence of conformational changes in a CD4-gp120-gp41 ternary complex as has been suggested in many reports (16, 44, 57, 62). Conformational changes of viral coat proteins have been shown to be pH dependent. Although infection by HIV-1 is not dependent on acidic pH (35, 48, 56), other viruses, e.g., influenza virus (55), Semliki Forest virus (61), and Sindbis virus (39), are dependent on exposure to mildly acidic pH for conformational changes which activate fusion proteins. Conformational changes in a CD4-gp120-gp41 ternary complex could very well be temperature dependent, as receptor binding (23, 39, 59) and membrane fusion (26) are both affected by temperature. Thermodynamic analysis of sCD4 binding to HIV-1 virions (41) supports this concept of temperature-dependent dynamic interactions between CD4 and gp120-gp41. This group demonstrated that high- and low-affinity binding of sCD4 to virions occurred at 37 and 4°C, respectively, and that the minimum temperature for both sCD4 affinity transition and shedding of gp120 ranged between 20 and 25°C. Alternatively, in a transiently gp120-expressing cell model (14), the affinity of sCD4 for cell-bound gp120-gp41 at 4 or 37°C was not significantly different, but the rate of reaching equilibrium was much slower at 4°C than at 37°C. Similar findings were demonstrated with another system (40), and the slow rate of reaching equilibrium at 4°C (versus 37°C) was attributed primarily to a slow rate of dissociation. The variations between different systems indicate that receptor clustering or multimeric molecular interaction could further complicate the issue of binding events. Thus far, no satisfactory system has been described for affinity and kinetics analyses of binding between cell-bound gp120 and CD4 molecules. Regardless of the effects of temperature on the apparent affinity or kinetics of binding events, receptor binding-initiated conformational changes are likely to be temperature dependent as has been demonstrated for Sindbis virus fusion proteins (39).

An important question which remains, however, is why 22°C is nearly nonpermissive for syncytium formation. Studies on lateral diffusion of transmembrane glycoproteins have shown that the rate of diffusion decreases with temperature (50), but the mobility of receptors for clustering is retained even at 4°C (5). Furthermore, pinocytosis, a process dependent on membrane fusion, can occur below 16°C, and a

threshold for receptor-mediated endocytosis has been observed at 15 to 17°C (25, 32, 33). These findings suggest that neither immobility of the CD4-gp120-gp41 ternary complex nor a nonspecific blockade of membrane fusion can explain the blockade of syncytium formation observed at 22°C. This observation suggests that there is an additional or alternative temperature-sensitive driving force for syncytium formation which may involve conformational change-mediated exposure and proteolytic cleavage of the V3 loop of gp120 (10).

In conclusion, our experiments have provided additional data which indicate that shedding of gp120 is not an integral step in cell-to-cell fusion mediated by HIV-1 envelope glycoproteins. We have also shown that CD4 may, analogously to sCD4, induce shedding of gp120 and that a metastable intermediate of fusion activation of the CD4-gp120-gp41 ternary complex may form following binding. Finally, additional or alternative conformational changes in the CD4-gp120-gp41 ternary complex probably are required for fusion.

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REFERENCES

1. **Allan, J. S.** 1991. Receptor-mediated activation of immunodeficiency viruses in viral fusion. Science **252:**1322–1322.
2. **Allan, J. S.** 1991. Receptor-mediated activation events in HIV/SIV viral entry, p. 133–156. *In* W. Koff, F. Wong-Staal, and R. C. Kennedy (ed.), AIDS research review, vol. 2. Marcel Dekker, New York.
3. **Allan, J. S., J. Strauss, and D. W. Buck.** 1990. Enhancement of SIV infection with soluble receptor molecules. Science **247:**1084–1088.
4. **Allan, J. S., E. M. Whitehead, K. Strout, M. Short, P. Kanda, T. K. Hart, and P. J. Bugelski.** 1992. Strong association of SIV_{agm} envelope glycoprotein heterodimers: possible role in receptor-mediated activation. AIDS Res. Hum. Retroviruses **8:**2011–2020.
5. **Anderson, C. M., G. N. Georgiou, I. E. G. Morrison, G. Y. W. Stevenson, and R. J. Cherry.** 1992. Tracking of cell surface receptors by fluorescence digital imaging microscopy using a charge-coupled device camera. Low-density lipoprotein and influenza virus receptor mobility at 4°C. J. Cell Sci. **101:**415–425.
6. **Berger, E. A., J. D. Lifson, and L. E. Eiden.** 1991. Stimulation of glycoprotein gp120 dissociation from the envelope glycoprotein complex of human immunodeficiency virus type 1 by soluble CD4 and CD4 peptide derivatives: implications for the role of the complementarity-determining region 3-like region in membrane fusion. Proc. Natl. Acad. Sci. USA **88:**8082–8086.
7. **Berger, E. A., J. R. Sisler, and P. A. Earl.** 1992. Human immunodeficiency virus type 1 envelope glycoprotein molecules containing membrane fusion-impairing mutations in the V3 region efficiently undergo soluble CD4-stimulated gp120 release. J. Virol. **66:**6208–6212.
8. **Bosch, M. L., P. L. Earl, K. Fargnoli, S. Picciafuoco, F. Giombini, F. Wong-Staal, and G. Franchini.** 1989. Identification of the fusion peptide of primate immunodeficiency viruses. Science **244:**694–697.
9. **Clapham, P. R., A. McKnight, and R. A. Weiss.** 1992. Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: induction and enhancement by soluble CD4. J. Virol. **66:**3531–3537.
10. **Clements, G. J., M. J. Price-Jones, P. E. Stephens, C. Sutton, T. F. Schulz, P. R. Clapham, J. A. McKeating, M. O. McClure, S. Thomson, M. Marsh, J. Kay, R. A. Weiss, and J. P. Moore.** 1991. The V3 loops of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion? AIDS Res. Hum. Retroviruses **7:**3–16.
11. **Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:**763–767.
12. **Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature (London) **331:**82–84.
13. **Dimitrov, D. S., H. Golding, and R. Blumenthal.** 1991. Initial stages of HIV-1 envelope glycoprotein-mediated cell fusion monitored by a new assay based on redistribution of fluorescent dyes. AIDS Res. Hum. Retroviruses **7:**799–805.
14. **Dimitrov, D. S., K. Hillman, J. Manischewitz, R. Blumenthal, and H. Golding.** 1992. Kinetics of soluble CD4 binding to cells expressing human immunodeficiency virus type 1 envelope glycoprotein. J. Virol. **66:**132–138.
15. **Dimitrov, D. S., R. L. Willey, M. A. Martin, and R. Blumenthal.** 1992. Kinetics of HIV-1 interactions with sCD4 and $CD4^+$ cells: implications for inhibition of virus infection and initial steps of virus entry into cells. Virology **187:**98–406.
16. **Eiden, L. E., and J. D. Lifson.** 1992. HIV interactions with CD4: a continuum of conformations and consequences. Immunol. Today **13:**201–206.
17. **Fisher, R. A., J. M. Bertonis, W. Weier, V. A. Johnson, D. S. Costopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsh, R. T. Schooley, and R. A. Flavell.** 1988. HIV infection is blocked in vitro by recombinant soluble CD4. Nature (London) **331:**76–78.
18. **Freed, E. O., D. J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc. Natl. Acad. Sci. USA **87:**4650–4654.
19. **Hart, T. K., R. Kirsh, H. Ellens, R. W. Sweet, D. M. Lambert, S. R. Petteway, J. Leary, and P. J. Bugelski.** 1991. Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. Proc. Natl. Acad. Sci. USA **88:**2189–2193.
20. **Healey, D., L. Dianda, J. P. Moore, J. S. McDougal, M. J. Moore, P. Estess, D. Buck, P. D. Kwong, P. C. L. Beverley, and Q. J. Sattentau.** 1990. Novel anti-CD4 monoclonal antibodies separate human immunodeficiency virus infection and fusion of $CD4^+$ cells from virus binding. J. Exp. Med. **172:**1233–1242.
21. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On entry of Semliki forest virus into BHK-21 cells. J. Cell Biol. **84:**404–420.
22. **Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H.-C. Chang, F. R. Siliciano, T. Dorfman, B. Walker, J. Sodoroski, and E. L. Reinherz.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature (London) **331:**78–81.
23. **Johnstone, R. W., S. M. Andrew, M. P. Hogarth, G. A. Pietersz, and I. F. C. McKenzie.** 1990. The effect of temperature on the binding kinetics and equilibrium constants of monoclonal antibodies to cell surface antigens. Mol. Immunol. **27:**327–333.
24. **Jonak, Z. L., R. K. Clark, D. Matour, S. Trulli, R. Craig, E. Henri, E. V. Lee, R. Greig, and C. Debouck.** 1993. A human lymphoid recombinant cell line with functional human immunodeficiency virus type 1 envelope. AIDS Res. Hum. Retroviruses **9:**23–32.
25. **Kielian, M. C., and Z. A. Cohn.** 1980. Phagosome-lysosome fusion: characterization of intracellular membrane fusion in mouse macrophages. J. Cell Biol. **85:**754–765.
26. **Kinchington, D., W. Barker, S. Galpin, and K. Apostolov.** 1992. Temperature enhancement of syncytium formation by HIV and Sendai virus. J. Med. Virol. **36:**44–48.
27. **Kirsh, R., T. K. Hart, H. Ellens, J. Miller, S. A. Petteway, D. M. Lambert, J. Leary, and P. J. Bugelski.** 1990. Morphometric analysis of recombinant soluble CD4 mediated release of the envelope glycoprotein gp120 from HIV-1. AIDS Res. Hum.

Retroviruses 6:1215–1218.
28. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. A. Haseltine, and J. Sodroski.** 1987. Functional regions of the human immunodeficiency virus envelope glycoprotein. Science **237**:1351–1355.
29. **Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon.** 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell **50**:975–985.
30. **Lifson, J. D., M. B. Finberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature (London) **323**:725–728.
31. **Lifson, J. D., G. R. Reyes, M. G. McGrath, B. S. Stein, and E. G. Engleman.** 1986. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. Science **232**:1123–1127.
32. **Manara, F. S., and D. L. Schneider.** 1985. The activation of the human neutrophil respiratory burst occurs only at temperatures above 17°C: evidence that activation requires membrane fusion. Biochem. Biophys. Res. Commun. **132**:696–701.
33. **Marsh, M., and A. Helenius.** 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. **142**:439–454.
34. **Marshall, W. L., D. C. Diamond, M. M. Kowalski, and R. W. Finberg.** 1992. High level of surface CD4 prevents stable human immunodeficiency virus infection of T-cell transfectants. J. Virol. **66**:5492–5499.
35. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7**:513–518.
36. **McDougal, J. S., M. S. Kennedy, J. N. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV-III/LAV to $T4^+$ cells by a complex of the 110K viral protein and the T4 molecule. Science **231**:382–385.
37. **McKeating, J. A., J. Cordel, C. J. Dean, and P. Balfe.** 1992. Synergistic interaction between ligands binding to the CD4 binding site and V3 domain of human immunodeficiency virus type 1 gp120. Virology **191**:732–742.
38. **McKeating, J. A., and R. A. Willey.** 1989. Structure and function of the HIV envelope. AIDS **3**:S35–S41.
39. **Meyer, W. J., S. Gidwitz, V. K. Ayers, R. J. Schoepp, and R. E. Johnston.** 1992. Conformational alteration of Sindbis virion glycoproteins induced by heat, reducing agents, or low pH. J. Virol. **66**:3504–3513.
40. **Moebius, U., L. K. Clayton, S. Abraham, S. C. Harrison, and E. L. Reinherz.** 1992. The human immunodeficiency virus gp120 binding site on CD4: delineation by quantitative equilibrium and kinetic binding studies of mutants in conjunction with a high-resolution CD4 atomic structure. J. Exp. Med. **76**:507–517.
41. **Moore, J. P., and P. J. Klasse.** 1992. Thermodynamic and kinetic analysis of sCD4 binding to HIV-1 virions and of gp120 dissociation. AIDS Res. Hum. Retroviruses **8**:443–449.
42. **Moore, J. P., J. A. McKeating, W. A. Norton, and Q. J. Sattentau.** 1991. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4. J. Virol. **65**:1133–1140.
43. **Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau.** 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science **250**:1139–1142.
44. **Moore, J. P., J. Q. J. Sattentau, P. J. Klasse, and L. C. Burkly.** 1992. A monoclonal antibody to CD4 domain 2 blocks soluble CD4-induced conformational changes in the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and HIV-1 infection of $CD4^+$ cells. J. Virol. **66**:4784–4793.
45. **Panataleo, G., L. Butini, C. Graziosi, G. Poli, S. M. Schnittman, J. J. Greenhouse, J. I. Gallin, and A. S. Fauci.** 1991. Human immunodeficiency virus (HIV) infection in $CD4^+$ T lymphocytes genetically deficient in LFA-1: LFA-1 is required for HIV-mediated cell fusion but not for viral transmission. J. Exp. Med. **173**:511–514.
46. **Rieber, E. P., C. Federle, C. Reiter, S. Krauss, L. Gurtler, J. Eberle, F. Deinhardt, and G. Riethmüller.** 1992. The monoclonal antibody M-T413 inhibits cellular infection with human immunodeficiency virus after viral attachment to the cell membrane: an approach to postexposure prophylaxis. Proc. Natl. Acad. Sci. USA **89**:10792–10796.
47. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174**:407–415.
48. **Sinangil, F., A. Loyter, and D. J. Volsky.** 1988. Quantitative measurement of fusion between human immunodeficiency virus and cultured cells using membrane fluorescence dequenching. FEBS Lett. **239**:88–92.
49. **Scudiero, D. A., R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, and M. R. Boyd.** 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. **48**:4827–4833.
50. **Scullion, B. F., Y. Hou, L. Puddington, J. K. Rose, and K. Jacobson.** 1987. Effects of mutations in three domains of the vesicular stomatitis viral glycoprotein on its lateral diffusion in the plasma membrane. J. Cell Biol. **105**:69–75.
51. **Sekigawa, I., S. M. Chamow, J. E. Groopman, and R. A. Byrn.** 1990. CD4-immunoadhesin, but not recombinant soluble CD4, blocks syncytium formation by human immunodeficiency virus type 2-infected lymphoid cells. J. Virol. **64**:5194–5198.
52. **Skehel, J., P. Bayley, E. Brown, S. Martin, M. Waterfield, J. Whit, I. Wilson, and D. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79**:968–972.
53. **Slepushkin, V. A., S. M. Andreev, M. V. Sidorova, G. B. Melikyan, V. B. Grigoriev, V. M. Chumakov, A. E. Grinfeldt, R. A. Manukyan, and E. V. Karamov.** 1992. Investigation of human immunodeficiency virus fusion peptides. Analysis of interrelations between their structure and function. AIDS Res. Hum. Retroviruses **8**:9–18.
54. **Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman, and D. J. Capon.** 1987. Blocking of HIV-1 infectivity by a soluble secreted form of the CD4 antigen. Science **238**: 1704–1707.
55. **Stegmann, T., J. M. White, and A. Helenius.** 1990. Intermediates in influenza induced membrane fusion. EMBO J. **9**:4231–4241.
56. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49**:659–668.
57. **Szabo, G., Jr., P. S. Pine, J. L. Weaver, P. E. Rao, and A. Aszalos.** 1992. CD4 changes conformation upon ligand binding. J. Immunol. **149**:3596–3604.
58. **Thali, M., C. Furman, E. Helseth, H. Repke, and J. Sodroski.** 1992. Lack of correlation between soluble CD4-induced shedding of the human immunodeficiency virus type 1 exterior envelope glycoprotein and subsequent membrane fusion events. J. Virol. **66**:5516–5524.
59. **Thali, M., U. Olshevsky, C. Furman, D. Gabuzda, J. Li, and J. Sodroski.** 1991. Effects of changes in gp120-CD4 binding affinity on human immunodeficiency virus type 1 envelope glycoprotein function and soluble CD4 sensitivity. J. Virol. **65**:5007–5012.
60. **Traunecker, A., W. Luke, and K. Karjalainen.** 1988. Soluble CD4 molecules neutralize human immunodeficiency virus type 1. Nature (London) **331**:84–86.
61. **Wahlberg, J. M., R. Bron, J. Wilschut, and H. Garoff.** 1992. Membrane fusion of Semliki Forest virus involves homotrimers of the fusion protein. J. Virol. **66**:7309–7318.
62. **Walker, L., D. Wilk, J. O'Brien, J. Habeshaw, and A. Dalgleish.** 1992. Localized conformational changes in the N-terminal domain of CD4 identified in competitive binding assay of monoclonal antibodies and HIV-1 envelope glycoprotein. AIDS Res. Hum. Retroviruses **8**:1083–1090.
63. **Werner, A., G. Winskowsky, and R. Kurth.** 1990. Soluble CD4 enhances simian immunodeficiency virus SIV_{agm} infection. J. Virol. **64**:6252–6256.