Single-Molecule Analysis of Human Immunodeficiency Virus Type 1 gp120-Receptor Interactions in Living Cells

Melissa I. Chang,¹ Porntula Panorchan,¹ Terrence M. Dobrowsky,¹ Yiider Tseng,¹ and Denis Wirtz,^{1,2*}

*Department of Chemical and Biomolecular Engineering¹ and Department of Materials Science and Engineering,²
The Johns Hopkins University, 3400 N. Charles St., Baltimore, Maryland 21218*

Received 15 June 2005/Accepted 27 August 2005

A quantitative description of the binding interactions between human immunodeficiency virus (HIV) type 1 envelope glycoproteins and their host cell surface receptors remains incomplete. Here, we introduce a single-molecule analysis that directly probes the binding interactions between an individual viral subunit gp120 and a single receptor CD4 and/or chemokine coreceptor CCR5 in living cells. This analysis differentiates single-molecule binding from multimolecule avidity and shows that, while the presence of CD4 is required for gp120 binding to CCR5, the force required to rupture a single gp120-coreceptor bond is significantly higher and its lifetime is much longer than those of a single gp120-receptor bond. The lifetimes of these bonds are themselves shorter than those of the P-selectin/PSGL-1 bond involved in leukocyte attachment to the endothelium bonds during an inflammation response. These results suggest an amended model of HIV entry in which, immediately after the association of gp120 to its receptor, gp120 seeks its coreceptor to rapidly form a new bond. This “bond transfer” occurs only if CCR5 is in close proximity to CD4 and CD4 is still attached to gp120. The analysis presented here may serve as a general framework to study mechanisms of receptor-mediated interactions between viral envelope proteins and host cell receptors at the single-molecule level in living cells.

Current antiviral therapies for treatment of human immunodeficiency virus type I (HIV-1) infection have greatly reduced the mortality and morbidity associated with HIV/AIDS. These treatments consist primarily of protease inhibitors that prevent viral maturation and reverse transcriptase inhibitors that block synthesis of proviral DNA. However, because of drug resistance (12), complementary strategies targeting virus-cell interactions before HIV-1 entry are currently under intense examination (8). While a structure-based model of interaction between HIV-1 envelope glycoproteins and host cell receptors is relatively well established (6, 22), a quantitative description of how linkages between cell and virus are formed to generate a functional junction remains incomplete. A quantitative description of the binding interactions between HIV-1 and cell surfaces is needed to further elucidate the molecular mechanisms of viral entry as well as potentially improve current HIV-1 entry inhibitors (8, 30, 31, 36).

The current qualitative picture of HIV-1 entry consists of sequential pairwise molecular interactions. HIV-1 targets primarily T lymphocytes and macrophages by initiating sequential binding interactions between CD4 receptors on these host cells and a surface subunit of the HIV-1 exterior envelope (Env) glycoprotein, gp120 (5). Binding of gp120 to CD4 promotes a conformational change in gp120, which mediates its binding to a cellular chemokine coreceptor: CCR5 for R5-tropic viruses (26, 33) and CXCR4 for X4-tropic viruses (9). Here, we focus on CCR5, which is also targeted by drug developers as the most promising site because individuals who naturally lack CCR5 due to the $\Delta 32$ polymorphism are highly resistant to

HIV infection, with no obvious immune deficits (23, 35). The CXCR4 coreceptor does not become involved until advanced stages of the infection (7, 37); CXCR4 use occurs in about half of individuals who progress to AIDS. Binding of gp120 to its coreceptor triggers a further conformational change of gp120, which exposes a previously buried hydrophobic fusion domain located at the N terminus of the transmembrane subunit of Env, gp41. Finally, binding of gp41 to the host cell surface promotes fusion between viral and cell lipid membranes, which mediates the entry of the genome-containing viral protein core into the host cell's cytoplasm to initiate the infection process (40, 43).

Crystallographic studies of gp120-receptor complexes have provided invaluable details about the structure of the HIV-1/cell binding interface at the atomic level (22, 33, 41, 42), but the actual details of the binding kinetics for virus-cell attachment remain poorly defined for living cells and at the single-molecule level. All kinetic measurements thus far have used of bulk methods and do not provide any information about the micromechanics of individual gp120-receptor bonds. For instance, the force required to break a single gp120-CD4 bond, its adhesion force, is unknown. Whether coreceptor CCR5 mechanically reinforces the primary gp120-CD4 bond is also unknown. Here, we used single-molecule force spectroscopy to probe quantitatively the kinetics and micromechanical properties of the principal bonds involved in the binding interactions between HIV-1 and its host cell. This single-molecule analysis does not require the use of soluble proteins or protein labeling, has exquisite sensitivity, accommodates living cells, and can unambiguously distinguish single-molecule binding events from multiple-molecule adhesion (i.e., avidity).

Using single-molecule analysis, we probed directly the binding interactions between an individual recombinant HIV-1 envelope glycoprotein subunit gp120 and an individual receptor

* Corresponding author. Mailing address: Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218. Phone: (410) 516-7006. Fax: (410) 516-5510. E-mail: wirtz@jhu.edu.

CD4 in the presence and absence of the coreceptor chemokine CCR5 in living cells. We find that, while the presence of CD4 is indeed required for CCR5 to bind to gp120, the force required to rupture the gp120-CCR5/CD4 bond and its lifetime are significantly higher than those for the gp120-CD4 bond. Together these results suggest a revised model for the initial binding interactions between HIV-1 and the host plasma membrane.

MATERIALS AND METHODS

Cell culture and reagents. GHOST cell lines (developed by V. KewalRamani and D. Littman) were grown in Dulbecco's modified Eagle's medium (American Type Culture Collection, Manassas, VA) supplemented with 10% fetal calf serum (American Type Culture Collection), 500 $\mu\text{g}/\text{ml}$ G418 (Sigma, St. Louis, MO), 100 $\mu\text{g}/\text{ml}$ penicillin-streptomycin (Sigma), 100 $\mu\text{g}/\text{ml}$ hygromycin (Sigma), and, for the coreceptor encoding cell line, 1 $\mu\text{g}/\text{ml}$ puromycin. HOS cell lines (developed by N. Landau) were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 1.0 $\mu\text{g}/\text{ml}$ puromycin. These cell lines were expanded on a 2- to 3-day cycle in a humidified 5% CO_2 -95% air incubator maintained at 37°C. For molecular force probe (MFP) experiments, during the passage of cells, 200 μl of a cell suspension of 1×10^6 cells/ml was added to each 60-mm tissue culture dish containing 5 ml of culture medium and was incubated overnight with 5% CO_2 at 37°C to allow cell spreading and restoration of normal cell morphology. Immediately before the MFP experiment, the medium was changed to serum-free medium containing HEPES (Invitrogen, Carlsbad, CA) to stabilize the pH while outside the incubator environment.

The cell surface CD4 complex monoclonal B4 has preferential recognition for cell surface CD4 over recombinant soluble CD4 (sCD4). It exerts apparent neutralizing activity against HIV by blocking access to the CD4 cell surface complex. Recombinant human sCD4 is the full-length extracellular domain of human CD4 (amino acids 1 to 370). This protein is reactive with HIV-1 gp120 and anti-CD4 monoclonal and polyclonal antibodies. Its glycosylation pattern is identical to that of the natural human protein. Recombinant HIV-1 BaL gp120 (molecular mass, 116 kDa) is the Env protein subunit of the macrophage-tropic virus that binds to CD4. This protein was prepared from HEK293 cells and purified by immunoaffinity chromatography using a monoclonal antibody specific for the conformational epitope of gp120. The GHOST/HOS cell lines, cell surface CD4 complex monoclonal B4, recombinant soluble CD4, and BaL gp120 were obtained through the NIH AIDS Research and Reference Reagent Program (NIAID).

Attachment of gp120 onto the cantilever tip. The cantilever tip (Veeco Instruments, Santa Barbara, CA) was first incubated in 1.0 mg/ml bovine serum albumin (BSA) (Sigma) overnight at 4°C. The cantilevers were then rinsed three times with phosphate buffer. BaL gp120 was then chemically cross-linked at room temperature to the cantilever tip with the membrane-impermeable agent bis(sulfosuccinimidyl)suberate (Pierce, Rockford, IL). Afterwards, the cantilevers were again washed three times with phosphate buffer and incubated overnight with 1.0 mg/ml BSA to block nonspecific binding. To verify that the functionality of gp120 was preserved in the cross-linking attachment, the tips were incubated with 1.0 mg/ml sCD4 with 5% CO_2 at 37°C. The cantilever tip with the cross-linked protein was then allowed to interact with cells plated on the bottom of a tissue culture dish. Upon retraction, no binding between the cell surface and the cantilever tip occurred (see more about this control experiment below).

MFP measurements. Single-molecule experiments were conducted using an MFP (Asylum Research, Santa Barbara, CA) (Fig. 1A). This instrument measures forces, with subpiconewton force resolution and millisecond time resolution, as a function of cantilever-sample separation distance, which is measured with subnanometer resolution. The MFP uses technology similar to the more conventional atomic force microscope by making use of a flexible cantilever tip that deflects in response to forces from between tip and cell surface.

Two outputs are generated from MFP experiments: the photodetector sensor output (in volts) and the linear variable differential transformer (LVDT) output. The photodetector sensor output is translated into cantilever deflection (in nanometers) by multiplying the sensor output by the inverse optical lever sensitivity, which is the inverse slope of the sensor output versus the LVDT output curve in the constant-compliance regimen. The LVDT output is translated into the tip-sample separation distance by taking the difference between the total cantilever movement and the displacement due to the applied force. The force measured from the cantilever deflection is calculated based on Hooke's law for linear elastic springs, $F = k\Delta x$, where F is the force (expressed in piconewtons),

FIG. 1. Schematic of the MFP used to measure binding interactions between individual HIV-1 gp120 molecules and receptors in a living host cell. (A) Schematic of the molecular force probe used in the measurements of gp120-receptor binding interactions. (B) A low density of recombinant BaL gp120 molecules are cross-linked to a cantilever, which is gently brought into contact with a cell. The cell is engineered to express either CD4 alone, coreceptor/chemokine CCR5 alone, or both CD4 and CCR5. The time of contact between cell and cantilever is kept to a minimum (<1 ms) to ensure mostly single-molecule interactions. The cantilever is subsequently pulled with a controlled reproach velocity. The time-dependent force applied on the bond between a gp120 molecule and a single cell receptor and the time-dependent deformation of the cell membrane-bound proteins are recorded simultaneously. The gp120-functionalized cantilever is shown in black when a gp120-receptor bond is subjected to a pulling force before rupture and in light gray after bond rupture.

k is the bending constant of the cantilever (in piconewtons per micrometer), and Δx is the deflection (in nanometers). The bending constant of the cantilever tip used, which in this case is the largest and softest triangular cantilever (nominal spring constant of 10 pN/ μm), was measured by the nondestructive thermal oscillation method before each new experiment. The time-dependent deflection of the cantilever was determined by laser deflection onto a photodetector and occurred at a rate of 1.0 kHz. Each cantilever was calibrated by the nondestructive thermal oscillation method before use (19); the cantilever had a mean bending constant of 10 pN/nm.

A 60-mm tissue culture dish containing receptor-expressing cells at 60% confluence was placed on the MFP stage. The protein-tethered cantilever was positioned directly above a single cell. The dwell time between the cantilever tip

and the cell was minimized to reduce the occurrence of multiple bond rupture events. As the cantilever retracted at a preset velocity, the force was recorded as a function of vertical displacement. Igor Pro 4.09 software (Wavemetrics, Inc., Lake Oswego, OR) was used to record rupture forces and loading rates from the force-versus-distance plots collected during experimentation. Rupture forces were binned corresponding to increments of 100 pN/s for loading rates of between 100 and 1,000 pN/s and 1,000 pN/s for loading rates of between 1,000 and 10,000 pN/s. The loading rate applied to protein-cell receptor interaction is equal to the product of the slope of the individual time-dependent force profiles (in piconewtons per micrometer) prior to rupture and the reproach velocity (in micrometers per second), also called retraction velocity.

RESULTS

Single gp120-CD4 bond force spectroscopy in living cells.

We used a single-molecule force spectroscopy assay to characterize the binding interactions between a single recombinant HIV-1 envelope glycoprotein BaL gp120 and its major receptor CD4 and coreceptor CCR5 on a living host cell. This assay measures rigorously kinetics and micromechanical parameters at the molecular level, thereby providing a wealth of biochemical and biophysical information about gp120-mediated bonds, which is not provided by traditional binding assays. Specifically, our assay measures the rupture force, dissociation rate, tensile strength, reactive compliance, and lifetime of the bond made by a single HIV-1 envelope glycoprotein subunit and individual receptors in living cells.

This assay uses an MFP (15), which consists essentially of a flexible triangular cantilever with tethered recombinant gp120 distributed at low density (Fig. 1). The cantilever is positioned over and gently brought into contact with a single living host cell and then pulled at a prescribed reproach velocity (Fig. 1A). The cell surface displays either the primary cell receptor for HIV-1 binding, CD4 (CD4⁺ GHOST parental cell line), the coreceptor/chemokine CCR5 (CD4⁺ HOS cell line), or both CD4 and CCR5 (GHOST Hi-5 cell line). The progressive deflection of the cantilever was recorded during the approach and reproach of the calibrated cantilever as a function of its vertical displacement until bond breakage(s) occurred (Fig. 2). These deflections were translated into force-displacement spectra such as those illustrated in Fig. 2. The spectrum was flat (constant force) when no rupture event occurred and displayed one (typically) or two (rarely) rupture events when bonds ruptured.

For each cell receptor assayed (CD4 and CCR5) and at each tested reproach velocity (5 to 45 $\mu\text{m/s}$), hundreds of force-displacement spectra were collected. From these force-displacement spectra, large data sets of rupture forces (i.e., the heights of the discrete force step at rupture [Fig. 2]) and loading rates were extracted and pooled into histograms (see Fig. 4). As shown below, this molecular-binding assay is specific, it allows us to report kinetic and biophysical properties of single-molecule interactions, and it leads to the direct measurement of rates of dissociation of a single gp120 molecule and individual CD4/coreceptors expressed in living cells at equilibrium (no applied force) and as a function of the applied force.

Distinguishing single-molecule interactions from multiple-molecule interactions. For long contact times between cell and cantilever and for high densities of gp120 molecules on the cantilever, gp120 molecules formed multiple bonds with the surface of the host cell. However, we were able to promote

FIG. 2. Force-distance spectra for gp120-CD4 interactions in living cells. Typical profiles of the time-dependent force acting on a gp120-CD4 bond under tension as a function of the time-dependent deformation of that bond are shown. From these force-distance spectra, the k_{off} rates of the bonds between HIV-1 envelope proteins and host cell receptors can be readily extracted. The probability of binding events between gp120 and its cell receptors depends on the contact time between cantilever and cell surface, the density of gp120 on the cantilever, and the approach force used to bring the cantilever in contact with the cell. These parameters are controlled to ensure mostly single-molecule interactions, as shown by single arrows in profiles 6 and 10 (numbered from the top) to indicate single-bond breakage. The two arrows in profile 1 show a rare case of double-bond breakage. Rupture force (in piconewtons) and loading rate (in piconewtons per second) are extracted from the height of the peak (in piconewtons) at the rupture point and from the product of the slope right before the peak (in piconewtons per micrometer) and the reproach velocity (in micrometers per second), respectively. Here, the reproach velocity was 15 $\mu\text{m/s}$. Experiments were conducted using CD4⁺ GHOST cells.

single gp120-receptor bond formation as opposed to multiple bonds by taking the following measures. (i) Cantilever and cell were allowed to interact for only a short time (<1 millisecond) to target the percentage of successful cell-cell interactions to be 10 to 30%. Based on Poisson distribution statistics, when 30% of cell-cantilever contacts result in gp120-CD4 binding events, >80% of these binding events involve a single bond, 15% involve double bonds, and <3% involve triple bonds (15, 16). Figure 2 illustrates force-displacement spectra when no binding event occurred (spectra 2 to 5 and 7 to 9 [numbered from the top]) and when distinct bond rupture events (spectra 1, 6, and 10) occurred. (ii) The force applied on the cell by the cantilever upon initial contact was lowered to reduce the surface area of contact between cantilever and cell surface (4, 34) and therefore reduce the probability of forming multiple bonds. (iii) The density of gp120 on the cantilever was made low by using short incubation times during cantilever preparation (see Materials and Methods).

We note that simple visual inspection of the force-displacement spectra readily distinguishes single-molecule interactions from (rare) multiple-molecule interactions. The former involve a single bond rupture event (marked by an abrupt force) (see, e.g., force-displacement spectra 6 and 10 in Fig. 2), whereas the latter involve multiple bond rupture events (spectrum 1 in

FIG. 3. The interactions between gp120-coated cantilevers and cell receptors are specific. Typical force-distance spectra for the binding association between individual gp120 and CD4 molecules in the absence (first spectrum) and presence (second spectrum) of an anti-CD4 function-blocking monoclonal antibody (mAb), for a cantilever without cross-linked gp120 (third spectrum), and for CD4⁺ cells in the presence of sCD4 (fourth spectrum) are shown. Experiments were conducted using CD4⁺ GHOST cells.

Fig. 2). We also verified that the rupture force histogram obtained at each reproach velocity (see Fig. 4B) displayed a unique well-defined peak as opposed to multiple quantized peaks that would occur if rupture force measurements for multiple bonds were inadvertently included in the data sets (4). Together these precautions ensured that the adhesive interactions between cell and cantilever recorded in our assay involved one bond, that nonspecific interactions between cantilever and cell were insignificant, and that only parameters characterizing single gp120-receptor binding interactions are reported here.

Single-molecule force spectroscopy measurements are specific. We verified that the gp120-cell interactions probed by our instrument were specific to CD4 by treating the cells with saturating amounts of an anti-CD4 function-blocking monoclonal antibody. We found that this cell treatment completely abrogated interactions between gp120 and CD4⁺ cells ($n = 300$ and 14 cells). A typical force-displacement spectrum for this case is shown in Fig. 3 (compare spectra 1 and 2 [numbered from the top]). We also found that cantilevers without cross-linked gp120 molecules and blocked with BSA elicited no bond rupture events ($n = 310$ and 10 cells) (see illustrative flat spectrum 3 in Fig. 3). Finally, we verified that, through competitive binding, the presence of saturating concentrations of sCD4 abrogated the binding interactions between gp120 molecules and CD4 ($n = 278$ for 12 cells) (see flat spectrum 4 in Fig. 3). Together, these controls ensure that the reported biochemical and biophysical parameters are specific to gp120-CD4 bonds.

The kinetic and micromechanical properties of a single gp120-CD4 bond. Our assay measures kinetics and mechanical parameters, including the dissociation rate, tensile strength,

and reactive compliance, which characterize the dynamics and micromechanics of a bond between gp120 and its host cell receptor and coreceptor expressed on the surface of living cells. These properties were evaluated using Bell's model for the three main molecular pairs (gp120-CD4, gp120-CCR5, and gp120-CCR5/CD4) known to mediate early R5 HIV-1 entry (3, 11). The Bell model relates the rupture force required to break a bond between two molecules to the applied loading rate. The same model has been successfully used by our group and others to characterize the binding interactions between ligands and the surface of living cells (e.g., selectin/PSGL-1 interaction) (1, 10, 15). The mean rupture force, f_m , is plotted as a function of the natural log of the loading rate, r_f (Fig. 4A):

$$f_m = \frac{k_B T}{x_\beta} \ln \left(\frac{r_f x_\beta}{k_{\text{off}}^0 k_B T} \right) \quad (1)$$

where $k_B = 1.38 \times 10^{-23}$ J/K is the Boltzmann constant and $T = 310$ K is the absolute temperature of the cell culture medium. By fitting the data with equation 1, we extracted the unstressed dissociation rate constant, k_{off}^0 , and the reactive compliance, x_β , of the gp120-CD4 bond. We note that while MFP measurements were collected under a nonzero loading rate, data extrapolation to a zero loading rate yields an unstressed (equilibrium) dissociation rate.

The experimental curve f_m versus r_f shows that the rupture force required to break a single gp120-CD4 bond does indeed grow linearly with the natural logarithm of the loading rate applied to that bond. However, this force-loading rate curve had a remarkable profile: the rupture force did not grow monotonically with loading rate. Instead, the rupture force grew with two different slopes before and after a characteristic loading rate of ~ 350 pN/s (Fig. 4A) (see more in Discussion). For comparison, the rupture force for the selectin/PSGL-1 bond grows uniformly over the same range of loading rates (15, 16).

Our force-spectroscopy measurements show that a gp120-CD4 bond could withstand mean forces of up to 26 pN before rupture, for a loading rate of 200 pN/s (Fig. 4A). The fitted f_m versus r_f curve using equation 1 yielded unstressed dissociation rate constants of 4.1 s^{-1} at low loading rates (< 350 pN/s) and 5.3 s^{-1} at high loading rates (> 350 pN/s), corresponding to bond lifetimes of 0.24 second and 0.19 second, respectively. This fitted data also determined that the reactive compliance of the gp120-CD4 bond was 0.14 nm at low loading rates and 0.11 nm at high loading rates. Following Jarzynski's reconstruction of an equilibrium free energy from single-molecule pulling measurements (18), the presence of two distinct slopes in the rupture force signifies that the free energy of interaction between gp120 and CD4 features two energy wells (Fig. 4C) (see more in Discussion).

The tensile strength, lifetime, and reactive compliance of the gp120-CCR5/CD4 bond are higher than those of the gp120-CD4 bond. Using living cells that expressed the coreceptor/chemokine CCR5 alone (no CD4), we applied the single-molecule spectroscopy method and the Bell model analysis described above to extract the tensile strength, lifetime, and reactive compliance of the gp120-CCR5/CD4 bond. Importantly, we detected no bond rupture events between gp120 and CCR5 in the absence of sCD4 (Fig. 5). Therefore, in agree-

FIG. 4. Single-molecule analysis of gp120-CD4 binding interactions. (A) Rupture force (in piconewtons) required to break a single gp120-CD4 bond as a function of the loading rate (in piconewtons per second) to which the bond is subjected. The unstressed dissociation rate k_{off}^0 and the reactive compliance x_B of the bond are calculated from this curve through Bell's model analysis (see text). This curve features a break at a characteristic loading rate of ~ 350 pN/s, which separates a regimen where the bond lifetime is long at low loading rates (see text). (B) Distributions of rupture forces required to break a single gp120-CD4 bond at different reproach velocities. (C) Schematic of the intermolecular potential of the gp120-CD4 interaction qualitatively based on data shown in panel A. Red represents the inner barrier potential of the gp120-CD4 interaction at high loading rates; blue represents the outer barrier potential at low loading rates. The width of each potential well is taken as the reactive compliance at low and high loading rates. Experiments were conducted using CD4⁺ GHOST cells.

ment with results from traditional binding assays, our assay suggests that gp120 and CCR5 cannot bind directly in the absence of CD4 in GHOST cells.

In contrast, in the presence of sCD4, we found that a significantly stronger interaction mediated the binding between

FIG. 5. Single-molecule analysis of gp120-CCR5 and gp120-CCR5/CD4 bonds. (A) Typical force-distance spectra for the gp120-CCR5 bond in the presence of sCD4, gp120-CCR5/CD4, and gp120-CCR5 in the absence of sCD4. (B) Distributions of rupture forces to break a single bond between a single gp120 molecule and the protein complex CCR5/sCD4 at different reproach velocities. (C) Rupture force for single gp120-CCR5 bonds (circles) in the presence of sCD4 and single gp120-CCR5/CD4 bonds (squares) as a function of the loading rate applied to those bonds. Here again, characteristic ruptures occur in the force-loading rate curves, at threshold loading rates of ~ 450 pN/s for the gp120-CCR5/sCD4 complex and ~ 650 pN/s for the gp120-CCR5/CD4 complex. All experiments were conducted using CD4⁺ CCR5⁺ GHOST Hi-5 cells.

gp120 and CCR5 compared to that between gp120 and CD4. Like the gp120-CD4 bond, the gp120-CCR5/sCD4 bond had a adhesion force that grew linearly with the natural log of the loading rate, at two different slopes before and after a higher characteristic loading rate of ~ 450 pN/s. The dissociation rate of the gp120-CCR5/sCD4 bond was significantly lower than that of the gp120-CD4 bond, i.e., 1.3 s^{-1} in the low-loading-rate regimen (< 450 pN/s) and 2.1 s^{-1} in the high-loading-rate regimen (> 450 pN/s), corresponding to bond lifetimes of 0.77 second and 0.48 second, respectively. The gp120-CCR5/CD4 bond could withstand mean forces of up to 29 pN before rupture, for a loading rate of 200 pN/s. Therefore, the force required to break a gp120-CCR5/sCD4 bond was similar to that required to break the bond between gp120 and CD4. Nevertheless, the gp120-CCR5/sCD4 bond was less dynamic and lasted significantly longer than the gp120-CD4 bond ($1/k_{\text{off}}^0 = 0.77 \text{ s}$ versus 0.24 s).

TABLE 1. Summary of the biochemical and biomechanical properties of gp120-CD4, gp120-CCR5/sCD4, and gp120-CCR5/CD4 compared to those of P-selectin/PSGL-1 and P-selectin/cancer cell LS174T ligand molecular bonds^a

Molecular pair	Dissociation rate, k_{off}^0 (s^{-1})		Tensile strength, F (pN)		Reactive compliance, x_{B} (nm)		Reference
	$<\gamma_c$	$>\gamma_c$	200 pN/s	2,000 pN/s	$<\gamma_c$	$>\gamma_c$	
gp120-CD4	4.1 ± 0.2	5.3 ± 0.2	26 ± 1	91 ± 3	0.14 ± 0.01	0.11 ± 0.01	This work
gp120-CCR5/CD4	0.7 ± 0.1	7.5 ± 0.7	35 ± 2	68 ± 3	0.24 ± 0.02	0.51 ± 0.02	This work
gp120-CCR5(sCD4)	1.3 ± 0.2	2.1 ± 0.2	29 ± 1	70 ± 3	0.28 ± 0.01	0.24 ± 0.01	This work
P-selectin/PSGL-1	0.22 ± 0.05		82	165	0.14 ± 0.01		16
P-selectin/LS174T ligand	2.78 ± 0.02		30	110	0.13 ± 0.01		15

^a γ_c indicates the threshold loading rate where a break occurred in the plots of rupture force versus loading rate; γ_c is 350 pN/s for the gp120-CD4 bond and 450 pN/s for the gp120-CCR5/CD4 and gp120-CCR5/sCD4 bonds (see text). Biochemical parameters for P-selectin-ligand bonds were evaluated over the entire range of loading rates. Values are means $\pm$ standard errors.

The dynamic interactions between gp120 and CCR5 in the presence of CD4 in living cells. Using GHOST His-5 cells expressing both CD4 and CCR5, we measured the binding kinetics between gp120 and the coreceptor/chemokine CCR5 in the presence of CD4. The protein complex gp120-CCR5/CD4 could withstand mean forces of up to 34 pN before rupture, for a loading rate of 200 pN/s. The rupture force measurements fitted to equation 1 yielded a k_{off}^0 rate of 0.7 s^{-1} at low loading rates ($<450 \text{ pN/s}$). Therefore, at low physiological loading rates, the tensile strength of the gp120-CCR5/CD4 bond was significantly higher and its lifetime (1.43 seconds) was significantly longer than those of the gp120-CD4 bond (0.24 second). The treatment of the cells with function-blocking monoclonal antibodies and small-molecule inhibitors against both CD4 and CCR5 eliminated all specific interactions between cells and the gp120-coated cantilever tip. Further, we verified that the measurements of the force required to break the gp120-CD4 bond obtained from GHOST Hi-5 cells expressing both CD4 and CCR5 receptors in the presence of a function-blocking antibody against CCR5 led to the same kinetic and mechanical Bell parameters as those obtained with the parental GHOST cell line (CD4 only) (data not shown).

DISCUSSION

We have described a single-molecule analysis of the binding interactions between HIV-1 gp120 and its major cell receptor CD4 and coreceptor CCR5. Our approach allows us to obtain not only familiar kinetic parameters, such as dissociation rate and bond lifetime (albeit at the single-molecule level), but also fundamental micromechanical properties of molecular bonds, such as rupture force and reactive compliance, which describe the mechanical strength of gp120-receptor bonds.

Previous structural and biochemical studies suggest that the functional forms of CD4 and CCR5 are monomeric (38, 39), although the state of aggregation of CD4 may be cell type dependent (24, 25). One could imagine that, upon contact of gp120 with the plasma membrane, gp120 molecules could interact with a cluster of CD4 molecules at the same time. However, if CD4 receptors were to fortuitously form an aggregate for a moment, the rate of reproach of the cantilever away from the cell remains sufficiently low to detect the rupture of individual molecular pairs (see, e.g., Fig. 2). Multiple bonds would have to break simultaneously to be indistinguishable from sin-

gle bond rupture event. The lifetime of the bonds involving multiple copies of CD4 or gp120 would be substantially longer, and the rupture force histograms would display multiple quantized peaks (as opposed to the observed unique well-defined peak), if rupture forces for multiple bonds were inadvertently part of the data sets (4). We also note that our assay shows that the lifetimes of the tested bonds, gp120-CD4, gp120-CCR5/sCD4, and gp120-CCR5/CD4, depend critically on the force applied to those bonds, a property that is not captured by traditional assays.

To place our gp120-CD4 interaction measurements in context, we compare the kinetic and micromechanical properties of the gp120-CD4 bond with those of important molecular bonds involved in immunology and metastasis. Table 1 compares the dissociation rate (k_{off}^0) and reactive compliance (x_{B}), as well as the mean rupture forces of the gp120-CD4, gp120-CCR5/CD4, and gp120-CCR5(sCD4) bonds, with those of P-selectin/PSGL-1 and P-selectin/carcinoma ligand bonds. These bonds are involved in leukocyte-endothelium interactions during inflammation and in carcinoma-endothelium interaction during metastasis, respectively (21). The kinetic and micromechanical parameters k_{off}^0 , x_{B} , and rupture force were assayed at the single-molecule level, in living cells, using the same molecular force probe assay (15, 16). HIV infection occurs mostly in the lymph nodes, where forces acting on the virus and loading rates, to which gp120-CCR5/CD4 bonds are subjected, are expected to be low. In contrast, upon binding to the endothelium, leukocytes and cancer cells are continuously subjected to hemodynamic flows, which subject P-selectin/ligand bonds to large forces (20). At low loading rates, the tensile strength of all gp120-receptor/receptor bonds is much lower ($P < 0.0001$) than that of the P-selectin/PSGL-1 or P-selectin/carcinoma ligand bonds: much smaller forces are required to break a gp120-receptor/receptor bond than to break P-selectin/ligand bonds. Moreover, the equilibrium lifetimes of gp120-receptor/receptor bonds were much shorter than those of P-selectin/ligand bonds. This result indicates that in the presence of a low force, such as the random force due to the Brownian motion of the virus on the surface of its host cell (of the order $k_{\text{B}}T\eta/\tau$, where τ is bond lifetime, η is the medium viscosity, and $k_{\text{B}}T$ is the thermal energy), the time for a gp120-receptor/receptor bond to dissociate is much shorter ($P < 0.0001$) than the time to dissociate the P-selectin/ligand bonds.

The equilibrium interaction energy for the gp120-receptor

can be semiquantitatively obtained from nonequilibrium pulling experiments. Following Jarzynski's reconstruction (18), we find that the equilibrium free energy of interaction between gp120 and its coreceptor features two wells. This is in sharp contrast to the P-selectin/PSGL-1 bond involved in leukocyte attachment to the endothelium, which features only one slope and, therefore, whose free energy displays only one well. This implies that gp120 and its receptors have to overcome two energy wells in order to detach spontaneously from each other. The sum of the reactive compliances for these two wells can be interpreted as the effective length of reaction path during mechanical retraction and the widths of the inner and outer potential barriers characterizing the binding interaction between gp120 and receptors (Fig. 4C). The underlying structural origin of these two wells is unclear at this time. Nevertheless, we could speculate that it may stem from the considerable conformational flexibility within gp120 (27). This test of this hypothesis will have to await mutational studies in combination with our MFP assay.

Using previous K_d measurements (2, 17), we can estimate the association rate (k_{on}) of gp120 for CCR5 by using our k_{off}^0 measurements: it is about 500-fold higher than the association rate of gp120 for CD4 (k_{on} of $\sim 2 \mu\text{M/s}$ versus $\sim 0.004 \mu\text{M/s}$). Together with previous crystallographic data, the results obtained here by single-molecule force spectroscopy suggest revisions of the current model of the early steps leading to HIV-1 entry, at least in cultured GHOST cells. In those cells, CD4 is absolutely required for the binding interaction between gp120 and its receptors (e.g., CCR5) (although the CD4 requirement depends on the isolate and the type of cells [13, 14]). Indeed, in the absence of CD4, interactions between gp120 and host cells are completely abrogated, and CCR5 alone cannot engage CD4. Nevertheless, the association of gp120 with CCR5 (with the required presence of CD4) is much more favorable than the association of gp120 with CD4. This suggests that right after the association of gp120 with CD4, gp120 seeks CCR5 to rapidly form a new bond. This "bond transfer" occurs only if CCR5 is in close proximity to CD4 to allow, within a time equal to or smaller than the lifetime of the gp120-CD4 bond, for CCR5 to form with CD4 a bond with gp120. Given the short lifetime of the gp120-CD4 bond, if CCR5 is not in the close vicinity of CCR5, gp120 will detach rapidly from CD4 and no bond can be established between gp120 and CCR5. CD4 and CCR5 do not seem to interact directly (38), although this may depend on receptor density and the cell type-dependent organization of these receptors on the cell surface (28, 29, 32). Therefore, the requirement of the close proximity of CCR5 to CD4 for effective binding of HIV to its receptors does not seem to be regulated.

The assay and analysis presented here may serve as a general framework to study mechanisms of receptor-mediated interactions between viral envelope proteins and host cell receptors at the single-molecule level. In particular, this approach could be used to study the single-molecule kinetics and micromechanical properties of the bonds that R5X4 gp120 makes with receptors relative to R5 and X4 gp120 molecules.

ACKNOWLEDGMENTS

We thank our colleague Robert F. Siliciano for advice and support and Sean X. Sun for fruitful discussions. We also thank one of the reviewers for insightful suggestions.

This work was funded by NASA grant NAG9-1563 and NIH grant GM065835.

REFERENCES

- Alon, R., D. A. Hammer, and T. A. Springer. 1995. Lifetime of P-selectin-carbohydrate bond in response to tensile force in hydrodynamic flow. *Nature* **374**:539–542.
- Babcock, G. J., T. Mirzabekov, W. Wojtowicz, and J. Sodroski. 2001. Ligand binding characteristics of CXCR4 incorporated into paramagnetic proteoliposomes. *J. Biol. Chem.* **276**:38433–38440.
- Bell, G. I. 1978. Models for the specific adhesion of cells to cells. *Science* **200**:618–627.
- Benoit, M., D. Gabriel, G. Gerisch, and H. E. Gaub. 2000. Discrete interactions in cell adhesion measured by single-molecule force spectroscopy. *Nat. Cell Biol.* **2**:313–317.
- Bour, S., R. Geleziunas, and M. A. Wainberg. 1995. The human immunodeficiency virus type 1 (HIV-1) CD4 receptor and its central role in promotion of HIV-1 infection. *Microbiol. Rev.* **59**:63–93.
- Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433**:834–841.
- Connor, R. L., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau. 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1-infected individuals. *J. Exp. Med.* **185**:621–628.
- Doms, R. W., and D. Trono. 2000. The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev.* **14**:2677–2688.
- Dragic, T. 2001. An overview of the determinants of CCR5 and CXCR4 co-receptor function. *J. Gen. Virol.* **82**:1807–1814.
- Evans, E. 2001. Probing the relation between force–lifetime–and chemistry in single molecular bonds. *Annu. Rev. Biophys. Biomol. Struct.* **30**:105–128.
- Evans, E., and K. Ritchie. 1997. Dynamic strength of molecular adhesion bonds. *Biophys. J.* **72**:1541–1555.
- Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman, and R. F. Siliciano. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**:1295–1300.
- Gorry, P. R., M. Churchill, S. M. Crowe, A. L. Cunningham, and D. Gabuzda. 2005. Pathogenesis of macrophage tropic HIV-1. *Curr. HIV Res.* **3**:53–60.
- Gorry, P. R., J. Taylor, G. H. Holm, A. Mehle, T. Morgan, M. Cayabyab, M. Farzan, H. Wang, J. E. Bell, K. Kunstman, J. P. Moore, S. M. Wolinsky, and D. Gabuzda. 2002. Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. *J. Virol.* **76**:6277–6292.
- Hanley, W., O. McCarty, S. Jadhav, Y. Tseng, D. Wirtz, and K. Konstantopoulos. 2003. Single molecule characterization of P-selectin/ligand binding. *J. Biol. Chem.* **278**:10556–10561.
- Hanley, W. D., D. Wirtz, and K. Konstantopoulos. 2004. Distinct kinetic and mechanical properties govern selectin-leukocyte interactions. *J. Cell Sci.* **117**:2503–2511.
- Hoffman, T. L., G. Canziani, L. Jia, J. Rucker, and R. W. Doms. 2000. A biosensor assay for studying ligand-membrane receptor interactions: binding of antibodies and HIV-1 Env to chemokine receptors. *Proc. Natl. Acad. Sci. USA* **97**:11215–11220.
- Hummer, G., and A. Szabo. 2001. Free energy reconstruction from nonequilibrium single-molecule pulling experiments. *Proc. Natl. Acad. Sci. USA* **98**:3658–3661.
- Hutter, J. L., and J. Beccofer. 1993. Calibration of atomic-force microscope tips. *Rev. Sci. Instr.* **64**:1868–1873.
- Konstantopoulos, K., W. D. Hanley, and D. Wirtz. 2003. Receptor-ligand binding: 'catch' bonds finally caught. *Curr. Biol.* **13**:R611–R613.
- Konstantopoulos, K., S. Kukreti, and L. V. McIntire. 1998. Biomechanics of cell interactions in shear fields. *Adv. Drug Deliv. Rev.* **33**:141–164.
- Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**:648–659.
- Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**:367–377.
- Lynch, G. W., E. K. Slaytor, F. D. Elliott, A. Saurajen, S. G. Turville, A. J. Sloane, P. U. Cameron, A. L. Cunningham, and G. M. Halliday. 2003. CD4 is expressed by epidermal Langerhans' cells predominantly as covalent dimers. *Exp. Dermatol.* **12**:700–711.
- Lynch, G. W., A. J. Sloane, V. Raso, A. Lai, and A. L. Cunningham. 1999. Direct evidence for native CD4 oligomers in lymphoid and monocytoid cells. *Eur. J. Immunol.* **29**:2590–2602.
- Martin, K. A., R. Wyatt, M. Farzan, H. Choe, L. Marcon, E. Desjardins, J. Robinson, J. Sodroski, C. Gerard, and N. P. Gerard. 1997. CD4-independent binding of SIV gp120 to rhesus CCR5. *Science* **278**:1470–1473.

27. Myszka, D. G., R. W. Sweet, P. Hensley, M. Brigham-Burke, P. D. Kwong, W. A. Hendrickson, R. Wyatt, J. Sodroski, and M. L. Doyle. 2000. Energetics of the HIV gp120-CD4 binding reaction. *Proc. Natl. Acad. Sci. USA* **97**: 9026–9031.
28. Nguyen, D. H., B. Giri, G. Collins, and D. D. Taub. 2005. Dynamic reorganization of chemokine receptors, cholesterol, lipid rafts, and adhesion molecules to sites of CD4 engagement. *Exp. Cell Res.* **304**:559–569.
29. Percherancier, Y., B. Lagane, T. Planchenault, I. Staropoli, R. Altmeyer, J. L. Virelizier, F. Arenzana-Seisdedos, D. C. Hoessli, and F. Bachelier. 2003. HIV-1 entry into T-cells is not dependent on CD4 and CCR5 localization to sphingolipid-enriched, detergent-resistant, raft membrane domains. *J. Biol. Chem.* **278**:3153–3161.
30. Pierson, T. C., R. W. Doms, and S. Pohlmann. 2004. Prospects of HIV-1 entry inhibitors as novel therapeutics. *Rev. Med. Virol.* **14**:255–270.
31. Pohlmann, S., and R. W. Doms. 2002. Evaluation of current approaches to inhibit HIV entry. *Curr. Drug Targets Infect. Disord.* **2**:9–16.
32. Popik, W., T. M. Alce, and W. C. Au. 2002. Human immunodeficiency virus type 1 uses lipid raft-colocalized CD4 and chemokine receptors for productive entry into CD4⁺ T cells. *J. Virol.* **76**:4709–4722.
33. Rizzuto, C. D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P. D. Kwong, W. A. Hendrickson, and J. Sodroski. 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**:1949–1953.
34. Sako, Y., A. Nagafuchi, S. Tsukita, M. Takeichi, and A. Kusumi. 1998. Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. *J. Cell Biol.* **140**:1227–1240.
35. Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cogniaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart, and M. Parmentier. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**:722–725.
36. Shen, X., and R. F. Siliciano. 2000. Preventing AIDS but not HIV-1 infection with a DNA vaccine. *Science* **290**:463–465.
37. Simmons, G., J. D. Reeves, A. McKnight, N. Dejucq, S. Hibbitts, C. A. Power, E. Aarons, D. Schols, E. De Clercq, A. E. Proudfoot, and P. R. Clapham. 1998. CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages. *J. Virol.* **72**:8453–8457.
38. Steffens, C. M., and T. J. Hope. 2003. Localization of CD4 and CCR5 in living cells. *J. Virol.* **77**:4985–4991.
39. Steffens, C. M., and T. J. Hope. 2004. Mobility of the human immunodeficiency virus (HIV) receptor CD4 and coreceptor CCR5 in living cells: implications for HIV fusion and entry events. *J. Virol.* **78**:9573–9578.
40. Wyatt, R., E. Desjardins, U. Olshevsky, C. Nixon, J. Binley, V. Olshevsky, and J. Sodroski. 1997. Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein. *J. Virol.* **71**:9722–9731.
41. Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski. 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**:705–711.
42. Xiang, S. H., P. D. Kwong, R. Gupta, C. D. Rizzuto, D. J. Casper, R. Wyatt, L. Wang, W. A. Hendrickson, M. L. Doyle, and J. Sodroski. 2002. Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein. *J. Virol.* **76**:9888–9899.
43. Yuan, W., S. Craig, Z. Si, M. Farzan, and J. Sodroski. 2004. CD4-induced T-20 binding to human immunodeficiency virus type 1 gp120 blocks interaction with the CXCR4 coreceptor. *J. Virol.* **78**:5448–5457.