Supporting Information

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SI Discussion

HIV-1 envelope glycoproteins are noncovalently associated heterotrimers containing two subunits, a gp120 surface subunit that functions as a cap to prevent premature refolding of the metastable transmembrane subunit gp41 (1, 2). FCs consist of gp120–gp41 trimers associated with cell surface CD4 receptors and coreceptors in specific but uncertain stoichiometries and geometrical arrangements. The binding of gp120 to CD4 induces a conformational change in gp120 that exposes the critical V3 loop region of gp120 and enables coreceptor binding, thereby facilitating further assembly of the FC and additional conformational changes (2). These coreceptor-dependent conformational changes in gp120 further weaken its hold on gp41, thereby lowering the activation energy barrier that prevents gp41 refolding by ∼5 kcal/mole (3). Although this allows gp41 refolding to proceed at a greatly accelerated rate, the residual activation energy barrier is substantial for most HIV-1 isolates, which results in a slow and stochastic flux of virions over this obstacle (3, 4). Because this flux is slow and stochastic, the cohort of stably adsorbed virions complete the entry process over an ∼20-h period rather than in a synchronous wave (Figs. 2 and 3) (5, 6), thereby explaining the slow completion of entry during our kinetic assays.

The refolding of gp41 occurs in several steps, first by forming a three-stranded coil (3SC) that is subsequently slowly converted into a six-helix bundle (6HB) or "trimer of hairpins" that pulls the virus closely onto the cell and energetically drives membrane fusion (1, 7). T-20 is a peptide inhibitor that binds into the grooves in the 3SC intermediate, thereby arresting entry (1). Because its binding is essentially irreversible, the inactivation occurs with pseudo first order kinetics and with an efficiency determined by T-20 concentration and the cell surface lifetime of the 3SC (3, 5, 8).

Analyses of HIV-1 virion entry suggest that FCs reversibly equilibrate with unbound CD4 and coreceptors throughout the entry process to mediate conversion of 3SCs into 6HBs. First, when FC assembly is inhibited by reducing CD4 or CCR5 concentrations or by CCR5 mutations, the steady-state rate of entry is reduced and T-20 sensitivity is increased compared with conditions of CD4 and CCR5 excess (Fig. 2) (3, 5, 8). Because T-20 sensitivity is proportional to the cell surface lifetime of the susceptible 3SC intermediate, this suggests that gp41 refolding is slowed (3, 5, 8). Thus, when CD4 or CCR5 concentrations are limiting potential FCs are incompletely assembled at equilibrium and only a fraction are competent to mediate entry at any moment. On the contrary, if FCs were irreversible complexes, they would slowly assemble to completion in these conditions and entry would not plateau at a reduced steady-state rate. Secondly, CCR5 antagonists bind irreversibly to CCR5 and rapidly block further entry of cell attached virions (9). This would not be expected if FCs were stable because these antagonists and gp120 bind CCR5 in a mutually exclusive manner. Indeed, entry kinetics measured using coreceptor antagonists and T-20 are identical, consistent with other evidence that coreceptors are required for conversion of 3SCs to 6HBs (3, 5, 6, 8). Because 6HB formation energetically drives the final step of membrane fusion (7), escape from T-20 and from coreceptor antagonists only occurs at that final stage of entry. On the contrary, we know that FCs become fully assembled rapidly in JC.53 cells because the stochastic process of entry begins at a maximum rate within ∼15 min after the cultures are warmed to 37 °C (Figs. 2 and 3). If these fully assembled FCs were irreversible, all of the virions would become resistant to TAK779 at that early time, long before all of the virions escape from T-20. Thus, our data (6, 10) demonstrate that escape from TAK779 also occurs very late in the entry process, implying that FCs must form reversibly.

Because coreceptor antagonists do not always rapidly terminate membrane fusion in cell–cell syncytia assays, it has been suggested that FCs might be stable. However, fusion of cells expressing HIV-1 envelope glycoproteins with cells containing CD4 and coreceptors occurs after the cells slowly coalesce to form large regions of tightly adherent juxtaposed membranes (synapses) that restrict inhibitor access and accumulate numerous FCs, each of which having only a small probability of mediating fusion. These factors, which are specific for syncytia assays, prevent efficient entry inhibitor function. For these reasons it is generally believed that syncytia assays do not accurately mimic virus entry or provide a robust approach for understanding FC stabilities or functions.

In the conditions of our experiments, infectious virions persist on cell surfaces for many hours. For example, as mentioned above, kinetic entry assays done using membrane-impermeable T-20 appear identical to those done using the CCR5 antagonist TAK779 (6). In addition, 2G12 neutralization can be efficiently reversed many hours after virion adsorption onto susceptible cells (e.g., Fig. 3). Similarly, the membrane impermeable Nt peptide also efficiently activates entry many hours after the virions are adsorbed onto cells and the cultures are warmed to 37 °C (Fig. 4 and Fig. S1). In fact, the number of infectious virions that enter after 12 h is not significantly dependent on whether Nt was added initially or at 12 h (Fig. S1). Therefore, infectious HIV-1 virions remain on cell surfaces for prolonged times and leave cell surfaces in a slow stochastic manner. This entry rate is reduced by 2G12.

SI Materials and Methods

Assay to Investigate the Effects of 2G12 and Other NAbs on Virion Adsorption to Target Cells. We investigated the effects of various neutralizing antibodies on HIV-1 adsorption onto cell surfaces by preincubating viruses with the following NMAbs (purchased from Polymun Scientific): 2G12 (100 μg/mL), b12 (15 μg/mL), or 2F5 (100 μg/mL) or with medium alone for 1 h at 37 °C. In addition, the HIV-IG pool (50 μg/mL) from chronically infected patients [obtained from the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH, contributed by North American Biologicals, Inc. (NABI, Boca Raton, FL) and National Heart Lung and Blood Institute] was tested using HIV-1_{SF162}, which is efficiently neutralized by that antibody. The virus-containing media were then incubated with 8 μg/mL DEAE-dextran–treated JC.53 cells for 30 min at 37 °C, and the cultures were rinsed, fixed, and processed for Gag p24 immunostaining and deconvolution microscopy as described below. Neutralization was verified in unfixed replicate cultures by adding TAK779 (15 μM final concentration, obtained from the ARRRP, contributed by Division of AIDS, NIAID, NIH) to terminate entry after the 30-min incubation period. Seventy-two hours later, infected foci were stained and counted.

Kinetic Analyses of HIV-1 Entry. To analyze the effects of NMAbs on entry kinetics, HIV-1 isolates were preincubated with NMAbs for 1 h or 16 h at 37 °C. HIV-1_{JRCSF} was preincubated with 50 μg/mL 2G12 or 15 μg/mL b12, whereas HIV-1_{SF162} was preincubated with 100 μg/mL 2G12. The virions were then spinoculated at 4 °C onto DEAE-dextran–treated JC.53 cultures as described (5, 6).

Postspinoculation washes and incubations at 37 °C were done in the presence of NMAbs as needed. Infections were stopped at specific times after warming by addition of TAK779. Entry kinetic assays were also performed in the presence of a partially inhibiting 250 nM TAK779 concentration and in HeLa-CD4 cells expressing the mutant coreceptor CCR5(G163R) [clone JGR.H3 (10)] or in cells that express a low amount of CCR5 [clone JC.10 (11)].

Slowing of HIV-1 entry in the latter kinetic assays was further investigated by analyzing T-20 sensitivities. Infections were done in the presence of serial 10-fold dilutions of T-20 (obtained from the ARRRP, contributed by Roche) ± 20 μg/mL 2G12 or 250 nM TAK779, using HIV-1_{JRCSF} and JC.53 target cells that were pretreated as above. We also investigated effects of low CCR5 expression levels and mutant CCR5 on HIV-1 T-20 sensitivities by using JC.10 and JGR.H3 cells as infection targets.

Inhibition of HIV-1_{JRCSF} by different concentrations of 2G12 and TAK779 was analyzed in JC.53 cultures that were pretreated or untreated with 8 μg/mL DEAE-dextran or polybrene as previously described (6).

Analyses of Fluorescently Labeled Virions. Immunofluorescent labeling of virions adsorbed by spinoculation onto DEAE-dextran–treated coverslips with adherent JC.53 cells or onto coverslips without cells was initiated by fixation in 3.7% formaldehyde for 30 min at room temperature, followed by rinsing two times with PBS (Gibco) and then permeabilizing in PBS containing 0.5% Nonidet P-40 for 30 min, followed by two rinses with PBS. Gag p24 was labeled by sequential incubations (1 h at room temperature) with an anti-p24 MAb (ARRRP, HIV-1 p24 hybridoma 183-H12-5C, contributed by B. Chesebro) followed by goat antimouse Alexa-594 (Invitrogen). 2G12 binding was detected by incubation with goat antihuman Alexa-488. Fluorescently labeled HIV-1 was observed using a Zeiss Axiovert 200M deconvolution microscope and images acquired using a 60× oil immersion objective and AxioVision software (release 4.6.3; Carl Zeiss) (6). Additional image acquisition and analysis of labeled virions was carried out using CytoVision software (version 3.93.1; Applied Imaging) with an Olympus BX61 microscope, CCD camera, and 100× oil-immersion objective. Images of fluorescently labeled virions were captured with camera settings adjusted so that the brightest particles were subsaturating. Optimal settings were determined on controls having maximal staining and were kept consistent for each sample within an experiment. Total fluorescence intensity of each virion particle in an image was obtained in the analysis window of CytoVision by selecting all particles in the image and then obtaining a fluorescence intensity value (i.e., the intensity of the selected object, as a percentage of the average saturation in the boxed image) and an area value (measured in pixels) for each particle in the image. The fluorescence intensity and area values for each virus particle were then multiplied to obtain total fluorescence per particle. Total particle fluorescence values were then summed and the average calculated for all virions assessed in an independent treatment group. Two to three complete image fields (~180–260 particles per image) were analyzed per experimental condition, representing a total of ~500 virions analyzed for each condition. In conditions of decreasing 2G12 gp120 binding, we observed that the average number of Alexa-488–labeled particles per field decreased with increasing time of incubation, as occurred in Figs. 3*E* and 4*C* (due to 2G12 dissociation or displacement). We corrected for this by multiplying total particle fluorescence values calculated for each time point by the total number of Alexa-488 stained particles at that time point normalized to the total number of stained particles at Time = 0 min. We then plotted fluorescence data versus time and added error bars (SEM of particle fluorescence intensity calculated without correction). Distributions of virion fluorescent intensities were generated using the binning function in Kaleidagraph (version 3.6.2; Synergy Software).

Analyses of 2G12 Reversibility. Reversibility of 2G12 inhibition was tested during the HIV-1 entry kinetics assay by washing out the NMAb at the 0-min time point immediately after spinoculation or after 60-min incubation of the cultures at 37 °C. Washout of 2G12 was confirmed by immunofluorescent antibody staining of virions adhered to DEAE-dextran–treated coverslips lacking cells with subsequent analysis using CytoVision software. Reversibility was also investigated by incubating virions with serial fivefold dilutions of 2G12 for 1 h at 37 °C, spinoculating this mixture onto duplicate cultures of JC.53 cells, and then washing one of the cultures in medium lacking 2G12 and incubating at 37 °C in the absence of 2G12, while the other culture was washed and incubated at 37 °C with appropriate 2G12 concentrations.

Assays to Test Coreceptor Dependency of 2G12 HIV-1 Inhibition. Our assay system uses an HIV-1_{JRSCF} variant (HIV-1_{JRCSF}-Ad) that was adapted to efficiently use chimeric CCR5(HHMH) possessing NIH/Swiss mouse extracellular loop 2 (3). Adaptive mutations in the V3 loop (S298N, F313L) and in V4 (N403S) that eliminates a glycosylation site allow HIV-1_{JRCSF}-Ad to efficiently use CCR5(HHMH). In addition, HIV-1_{JRCSF}-Ad infection of HeLa-CD4/CCR5(Δ18) cells (12), which express a mutant CCR5 lacking 18 N-terminal residues, is rapidly and efficiently triggered only when a 22-residue tyrosine-sulfated N-terminal CCR5 peptide (Nt) (American Peptide Company) is supplied *in trans* (3). To ascertain the effect of 2G12 on Nt-triggered infections of HeLa-CD4/CCR5(Δ18) cells, HIV-1_{JRCSF}-Ad was preincubated for 1 h at 37 °C with 4 μg/mL 2G12 or medium alone, and infections were then carried out in the presence of varying concentrations of Nt. To determine whether 2G12 inhibits a coreceptor-dependent step or an earlier step, we performed a kinetic assay by spinoculating HIV-1_{JRCSF}-Ad that had been pretreated for 1 h at 37 °C with 4 μg/mL 2G12 or with medium alone onto DEAE-dextran–treated HeLa-CD4/CCR5 (Δ18) cells with 100 μM Nt added immediately after spinoculation or after the cultures had been at 37 °C for 60 min.

Displacement of 2G12 by the competitive assembly of fusion complexes was assessed by spinoculating HIV-1_{JRCSF}-Ad that had been preincubated with 4 μg/mL 2G12 for 1 h at 37 °C onto DEAE–dextran–coated coverslips that lacked cells. The virions were washed with 2G12 containing media with subsequent incubations in media containing 2G12 alone or 2G12 + sCD4 (sCD4 obtained from Perkin-Elmer; 5 μg/mL final concentration) or 2G12 + sCD4 + Nt (Nt = 100 μM final concentration). Some wells were fixed immediately (0 min), whereas others were incubated at 37 °C and fixed at 60 or 120 min. 2G12–gp120 binding was then visualized and analyzed using the immunofluorescence assay and CytoVision as described above. To determine whether the 2G12 displacement we observed was due to gp120 shedding, we assessed the gp120 content of virions after 60-min incubation at 37 °C with 2G12 ± sCD4 + Nt using a sheep anti-gp120 antibody (D7324; Aalto Bio Reagents) followed by donkey antisheep Alexa-488 (Invitrogen). We obtained deconvoluted images using subsaturating exposures as described above and we then quantitated individual virion fluorescence intensities using an AxioVision automeasure module (AV4 Mod Automeasure; Carl Zeiss Microscopy).

Gp120 Structural Modeling. The gp120 crystal structure (PDB ID 2B4C) (13) including the V3 loop and N-linked glycosylation sites important for 2G12 binding was generated using the PyMOL Molecular Graphics system (version 1.2r1; Schrödinger) (14).

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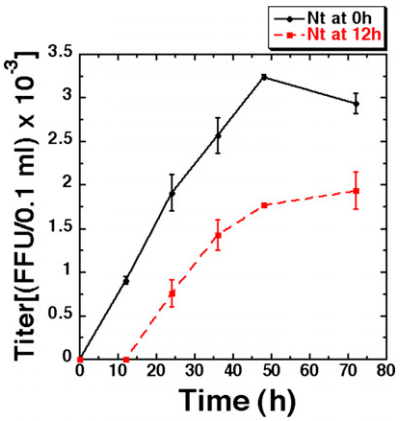

Fig. S1. HIV-1 entry can be triggered many hours after adsorption onto target cell surfaces. HIV-1_{JRCSF}-Ad, which efficiently infects HeLa-CD4/CCR5(Δ18) cells only in the presence of a 22-residue amino-terminal tyrosine-sulfated CCR5 peptide Nt was spinoculated onto DEAE-dextran–treated HeLa-CD4/CCR5(Δ18) cells at 4 °C and the cultures then warmed to 37 °C. Nt was then added to cultures (final concentration, 100 μM) 12 h postwarming, whereas control cultures received Nt immediately before warming. Kinetics of entry was monitored by TAK779 addition upon initial warming with continued additions at subsequent time points. Representative experiment is shown. Error bars are the range.

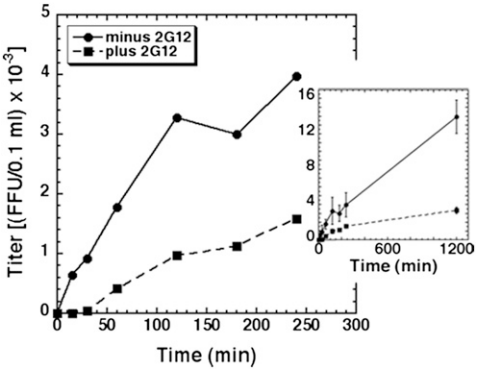

Fig. S2. 2G12 delays and slows entry of HIV-1_{SF162}. Virions preincubated at 37 °C for 1 h ± 100 μg/mL 2G12 were bound onto JC.53 cells by spinoculation and entry kinetics were measured. *Inset* shows data plotted to 1,200 min, whereas the larger graph plots initial kinetics to 240 min. A representative experiment of three is shown. Error bars are ± SD.

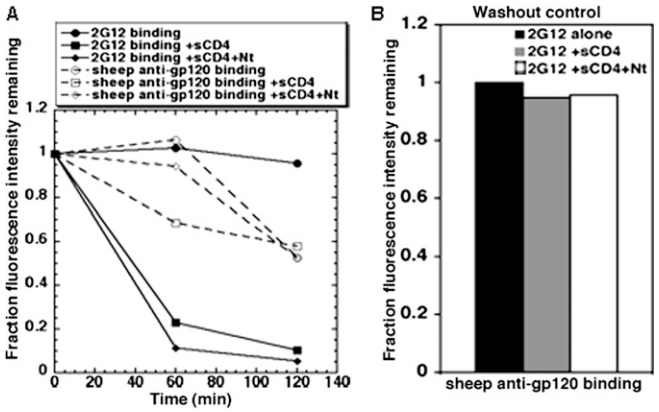

Fig. S3. Displacement of 2G12 from wild-type HIV-1_{JRCSF} by sCD4 ± Nt. (*A*) Similar to HIV-1_{JRCSF}-Ad, sCD4 and the amino terminal CCR5 peptide Nt compete with 2G12 for gp120 binding onto parental HIV-1_{JRCSF}. Virions preincubated with 2G12 (50 μg/mL) for 1 h at 37 °C were bound to coverslips by spinoculation. The virions were washed and media containing 2G12 alone or 2G12 + sCD4 (45 μg/mL) or 2G12 + sCD4 + Nt (Nt = 200 μM) were added to the wells, and some wells were fixed immediately (0 min), whereas others were incubated at 37 °C and fixed at 60 or 120 min, and subjected to immunofluorescence assay. Residual 2G12 was detected using goat antihuman Alexa-488 antibody. Virion-associated gp120 content was assessed in these same conditions using a sheep anti-gp120 antibody, which confirmed that 2G12 displacement was not due to gp120 shedding. (*B*) Additional gp120 shedding control. To evaluate the possibility that 2G12 and/or sCD4 might competitively interfere with the sheep anti-gp120 antibody, virions preincubated with 2G12 were treated as in *A*, except that after 60 min at 37 °C in the specified conditions, adhered virions were washed and then incubated for an additional 60 min at 37 °C in complete medium lacking any additives to allow ligand dissociations. The virions were then fixed and gp120 detected using sheep anti-gp120 antibody. Virion fluorescent intensities of deconvoluted images were quantitated using Automeasure in AxioVision (release 4.6.3).