Supplementary Information:

An allosteric rheostat in HIV-1 gp120 controls the stoichiometry of CCR5 required for membrane fusion

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Supplementary Experimental Procedures.

Construction, expression, and coreceptor activity of mutant CCR5s. Point mutations were introduced into the CCR5 ECL2 domain using the QuickChange (Stratagene) mutagenesis method. Cell surface expression was tested by transiently expressing pcDNA3.0 based vectors encoding wild-type or mutant CCR5s in 293T cells and performing an immunofluorescence assay using either the anti-CCR5 MAb, 2D7 (BD Biosciences), or a rabbit polyclonal Ab against the CCR5 N-terminus.

Peptide N-glycosidase digestion of wild-type and mutant CCR5s. For **Fig S1**, L-[^{35}S]methionine labeled extracts were prepared from transiently tranfected 293T cells by washing the cells in PBS and then lysing in solubilization buffer (0.75% CHAPSO, 0.25% cymal-5, 0.1M $(NH_4)_2SO_4$, 20mM Tris pH 8.5, 10% glycerol). CCR5s were immunoprecipitated from the cell extracts using the 2D7 MAb and protein A sepharose (Sigma-Aldrich, St. Louis, MO). The CCR5 immunoprecipitates were resuspended 1% SDS/PBS then further diluted into peptide N-glycosidase F digestion buffer (1% NP40/PBS) in the presence or absence of PNGase F (Roche, Palo Alto, CA, 1 U per sample) and incubated at 37° C for 4 h. The control and treated proteins were then fractionated by SDS-PAGE. The separated, radiolabeled proteins were transferred to MagnaGraph Nylon Transfer Membranes (Osmonics, Inc., Minnetonka, MN) and detected by autoradiography of the nylon membranes.

Supplementary Theoretical Considerations.

How are HIV-1 infection efficiencies altered by changes in CCR5 stoichiometries and membrane fusion kinetics?

(a) Effects of CCR5 Stoichiometries and Envelope Glycoprotein Redundancy on HIV-1 Infections.

To consider how viral differences in minimum CCR5 stoichiometries would affect entry, we initially assume that the infectious virions on cell surfaces interact with the saturating concentration of CD4 on our test cells to form complexes that contain **N** binding sites for CCR5(HHMH). If these binding sites were independent, the probability ($\mathbf{p_B}$) that any single binding site would be occupied at equilibrium is **[CCR5(HHMH)]/{[CCR5(HHMH)]+k }** and would equal 0.5 when **[CCR5(HHMH)]/ k = 1**, where **k** is the dissociation constant of the individual binding sites. Similarly, the probability ($\mathbf{p_U}$) that any single site would be unbound is **k /{[CCR5(HHMH)]+ k }**. This allows us to estimate $\mathbf{p_B}$ and $\mathbf{p_U}$ as functions of the **[CCR5(HHMH)]/ k** ratio. We then used the binomial theorem to estimate the probabilities ($\mathbf{P_{\geq i}}$) that individual virions on the cells would have CCR5(HHMH) stoichiometries ≥ specific integral values **i≤N** as a function of **[CCR5(HHMH)]/ k** ratios and assuming different values of **N** and a constant value of **k** for all binding sites. Specifically, the probability that the virions would have $\mathbf{P_{\geq 1} = 1-(p_U)^N}$. Moreover, the probability that the virions would have precisely **i** bound CCR5(HHMH)s would be given by the generic formula

$$\mathbf{P_i} = \begin{bmatrix} N \\ i \end{bmatrix} (\alpha^i)[1/(1+\alpha)]^N \qquad \textbf{(Eq S1)}$$

where **α=[CCR5(HHMH)]/ k.**

This allows us to calculate $\mathbf{P_{\geq 2}=P_{\geq 1}-P_1}$, and $\mathbf{P_{\geq 3}=P_{\geq 2}-P_2}$, etc. The resulting $\mathbf{P_{\geq 1}}$, $\mathbf{P_{\geq 2}}$, and $\mathbf{P_{\geq 3}}$ theoretical curves are plotted for an **N** value of 3 in **Fig S3**. Although other values of **N** could have been

used, the value of 3 was employed for purposes of illustration because the functional envelope glycoproteins are trimers and because our allosteric analyses in **Fig 5** support the use of this number. The curves in **Fig S3** demonstrate that the ensemble of complexes with CCR5(HHMH) stoichiometries ≥1 would form at lower concentrations and with less apparent cooperativity than ensembles of complexes that contain ≥2 or ≥3 CCR5(HHMH)s. This analysis shows how a change in minimum stoichiometry required for infection without any change in affinity of individual binding sites would strongly affect the efficiencies of assembling fusion-competent viral complexes on cell surfaces.

It is reasonable to assume that the viral envelope glycoproteins in the virus-cell junctions are gp120-gp41 trimers and that these trimers function either individually or in collaborating groups to mediate membrane fusion. However, some virions might conceivably form more than one independent junction complex with the cell, and each of these redundant complexes might potentially associate with a sufficient number of CCR5(HHMH)s to become fusion-competent. **Fig S3** shows an example of how the presence of a second junction complex at the virus-cell interface would influence the probability that the virus would be in a fusion-competent state. This factor causes the $\mathbf{P_{\geq 2}}$ curve to shift position toward lower values on the CCR5(HHMH) concentration axis, but has only a minor effect on its sigmoidal shape. Moreover, we do not believe that the adaptive mutations could function solely by increasing the number of redundant junctional complexes with the cell surfaces. In support of this conclusion, the wild-type and adapted envelope glycoproteins are synthesized, processed, and released from cells in similar amounts (**Fig S2**), in agreement with our previous analyses of these and other adaptive mutations [1].

(b) Kinetic control of HIV-1 infections. Some viruses use CCR5(HHMH) inefficiently even at saturating concentrations (see **Fig 4A**), clearly showing that efficiencies of infection do not depend solely on the probabilities that the virions associate with a sufficient number of CCR5(HHMH)s to form fusion-competent complexes. It has been demonstrated that HIV-1 virions diffuse into contact with cell surfaces

independently of their gp120 or the cellular coreceptor concentration [2; 3]. Consequently, viable virions that contact weakly susceptible cells must either predominantly dissociate from the cell surfaces or become inactivated. In contrast, virions that are more adapted or that contact cells having higher CCR5(HHMH) concentrations fuse more rapidly, and this allows them to escape the inactivation process more efficiently [4]. Thus, as we previously showed, infectious virions that contact HeLa-CD4/CCR5 cell surfaces are in a competitive race between an inactivation pathway that occurs at a uniform rate constant $\mathbf{k_d}$ for all HIV-1 isolates and a pathway for successful entry that requires coreceptors [4]. Moreover, the lag phase preceding the onset of viral entry is short compared to the time course for entry [4]. We therefore assume for our initial calculations that the cell surface virions form an equilibrium ensemble of complexes, and that a proportion **P** of these complexes are competent for membrane fusion, although we need not specify at this point whether the competent complexes are homogeneous or heterogeneous. Initially, we also assume that all competent complexes escape the inactivation phase by infecting at a uniform rate constant $\mathbf{k_i}$, although this will be modified as described below. These considerations imply that viable virions (**V**) leave the cell surface due to successful infection plus inactivation at a rate $\mathbf{dV/dt= -V(k_iP+k_d)}$, which can be integrated to give $\mathbf{V= V_0exp\{-(k_iP+k_d)t}$. Substitution of this into an equation for the rate of infection $\mathbf{di/dt= k_iPV}$ and integration throughout the time course of infection until all virions have left the cell surfaces, yields an estimate for the final level of infection $\mathbf{i = V_0k_ip/(k_iP+k_d)}$. This implies that $\mathbf{i/V_0}$, the efficiency of successful entry (**E**), would be

$$\mathbf{E= k_iP/(k_iP+k_d)} \qquad \textbf{(Eq S2)}$$

At high CD4 and CCR5 concentrations, all virions are in competent complexes and **P** equals one, and in this condition $\mathbf{E_{max}= k_i/(k_i+k_d)}$. The infectivity values ($\mathbf{i_{rel}}$) that we measure in any cell clone are normalized relative to the titer of the same virus measured in the optimally susceptible JC.53 cell clone that contains a large amount of both CD4 and CCR5. These $\mathbf{i_{rel}}$ values are used routinely in our work

because it is necessary to titer all of our viral preparations as a precondition for evaluating their infectivities in different cell clones. Moreover, when we prepare pseudotyped viruses with different envelopes in parallel transfections, they have similar titers in the optimally susceptible JC.53 cells [1; 5]. Thus, we are able to estimate efficiencies of infection only in a relative sense rather than on an absolute scale. Consequently, $\mathbf{i_{rel}=E/E_{JC53}}$ and $\mathbf{i_{rel\ max}=E_{max}/E_{JC53}}$.Therefore, the efficiency of infection at any CCR5 concentration normalized relative to the maximal efficiency at saturating CCR5 concentrations will be

$$\mathbf{E/E_{max}= i_{rel}/i_{rel\ max}= P(k_i+k_d)/(Pk_i+k_d)} \qquad \textbf{(Eq S3)}$$

Thus, although we cannot directly measure **E** values because we don't know the absolute number of viable virions $\mathbf{V_0}$ that contact the cells, it is lower than $\mathbf{i_{rel}}$ because $\mathbf{E_{JC53}}$ is considerably less than one. Indeed, our previous kinetic analysis suggested that $\mathbf{E_{JC53}}$ is approximately 0.2 [4]. Since the $\mathbf{i_{rel\ max}}$ value for the most adapted virus is approximately 0.4 in cells containing CCR5(HHMH) (**Table 1**), these considerations imply that $\mathbf{k_i \sim 0.09k_2}$ for that assay and is much lower for the unadapted viruses. These equations provide a framework for interpreting how the adaptive mutations influence the efficiencies of infection. Essentially, our results suggest that the adaptations increase the rate constant for entry $\mathbf{k_i}$ relative to the rate constant $\mathbf{k_d}$ of virus inactivation, thereby increasing $\mathbf{E_{max}}$ and enabling the virus to more efficiently and rapidly infect the cells. Moreover, by reducing the minimum CCR5(HHMH) stoichiometry required in the fusion complexes, the adaptations would also greatly increase **P** values especially at limiting CCR5(HHMH) concentrations as shown in **Fig S3**. Substituting the $\mathbf{P_{\geq i}}$ estimates from the previous section into equations **S2** and **S3** also enables us to estimate how the efficiencies of infection would be affected by viral differences in the $\mathbf{k_i/k_d}$ ratio. **Fig S4A** illustrates how the efficiencies of infection **E** would be expected to depend on the $\mathbf{k_i/k_d}$ ratio when $\mathbf{P_{\geq 1}}$ or $\mathbf{P_{\geq 2}}$, as determined using equation **S2**. Similarly, **Fig S4B** shows how the normalized efficiency ratio $\mathbf{E/E_{max}}$ would be expected to

depend on $\mathbf{k_i/k_d}$. The family of curves in **Fig S4A** and **S4B** resemble the experimental results in **Figs 4A**and **B** respectively, as well as the data in our previous papers [1; 6]. Importantly, although increasing the $\mathbf{k_i/k_d}$ ratio greatly increases the efficiency of infection (**Fig S4A**), it has only a slight effect on the normalized $\mathbf{E/E_{max}}$ values when $\mathbf{k_i \leq 0.5k_d}$ (**Fig S4B**), which is the case for our experiments as described above. Indeed, our results suggest that $\mathbf{k_i < 0.1k_d}$ in our experiments. When $\mathbf{k_i << k_d}$, equations **S2** and **S3** can be simplified to

$$\mathbf{E = k_i P/k_d} \qquad \textbf{(Eq S4)}$$

$$\mathbf{E/E_{max} = i/i_{rel\,max} = P} \qquad \textbf{(Eq S5)}$$

The above derivation can be modified based on our evidence that the ensemble of competent complexes on any cell contains components with distinct CCR5 stoichiometries that enter cells with different rate constants $\mathbf{k_i}$. Consequently, the cells with maximum CCR5 will be infected most rapidly with an efficiency estimated from (Eq **S4)** of $\mathbf{E_{max} = k_{i,max}/k_d}$. On any cell clone there will be a distribution of complexes and only those with at least a minimum number of CCR5s will be competent for infection. The probability that a virus on the cell would be in a competent complex would therefore be $\mathbf{P_{\geq J} = P_J + P_K + \ldots P_N}$, where **J** is the minumum number of CCR5s required and **N** is the total number of binding sites available on each adsorbed virion. As an example, the component of size **J** in the ensemble of competent complexes will infect with a specific efficiency determined by equation **S4** of $\mathbf{E_J = k_{i,J} P_J/k_d}$. This implies that the overall efficiency of infection for the ensemble would be the sum of efficiencies for the competent components. Consequently, we can write

$$\mathbf{i_{rel}/i_{rel,max}} = \sum_{J}^{N} \mathbf{P_J f_J} \approx \sum_{J}^{N} \mathbf{P_J(k_{i,J}/\,k_{i,max})} \qquad \textbf{(Eq S6)}$$

where $\mathbf{f_J}$ is the efficiency of infection by component **J** normalized relative to the efficiency of the maximally sized component in the ensemble.

(c) Implications of our activation free energy measurements. An aspect of uncertainty concerns the free energy barriers measured by our experiments (**Table 1**). In particular, recent evidence has implied that coreceptors stimulate all of the essential conformational changes in gp41 that result in membrane fusion, including both the formation of the 3SC coiled-coil that extends into the target cell membrane and its subsequent conversion into the stable 6HB[7]. Thus, both processes are inhibited by coreceptor antagonists. In contrast, earlier evidence had implied that the 3SCs can form to at least a small degree in the presence of CD4 in the absence of a coreceptor [8; 9; 10]. However, this was initially analyzed using only highly fusogenic laboratory-adapted HIV-1 variants that use CXCR4 as a coreceptor and are exceptionally susceptible to inactivation by sCD4. Since sCD4 irreversibly inactivates all HIV-1 isolates at high concentrations during prolonged incubations, it is unsurprising that it would eventually induce irreversible changes in gp41 including 3SC formation. In this context, we emphasize that our analysis applies specifically to the free energy barrier that controls the efficiency of infection, which is determined by competition between a viral inactivation or dissociation process and a coreceptor-dependent step(s) that enables the virus to escape [4]. One interpretation is that the inactivation might involve dissociation or endocytosis and that 3SC formation would fix the virus onto the cell surface and overcome the jeopardy. In that case, 6HB formation would occur subsequently and the efficiency of infection would not depend on its rate. Alternatively, the inactivation process might occur throughout the period preceding membrane fusion, in which case the efficiency of infection would depend strongly on the rate of the 3SC-to-6HB conformational change. Further evidence will be required to resolve these issues.

The free energy barriers determined by our measurements (**Table 1**) are in the range of ~5 kcal/mole for unadapted HIV-1_{JRCSF}. It is important to understand that these values pertain only to the allosteric free energy barriers that are imposed by gp120 and are overcome by associations with CCR5. Clearly this gp120-imposed barrier is much less than the total free energy barrier of ~24 kcal/mole that

limits fusion of lipid bilayer membranes [11]. While it is unlikely that such a large barrier could be overcome in a single step in a protein mediated process, it is evident that the membranes of virions and cells contain embedded proteins that may weaken the bilayer in local regions. Additionally, it is likely that the barrier is overcome by a multistep process that includes at least the following steps: (a) virus attachment onto the cell, (b) aggregation of CD4 and coreceptors into the complex resulting in exclusion of lipids and water, (c) insertion of gp41 fusion peptides into the cell membrane, and (d) 6HB formation. According to this idea, the step(s) influenced by the CCR5-induced allosteric transition in gp120 could critically control the fusion process in a manner compatible with our measurements despite other contributions to the overall energetics.

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Supplementary Figure Legends.

Supplementary Figure 1. CCR5 ECL2 mutations. (A) Alignment of human and murine (NIH/Swiss mouse) CCR5 ECL2 amino acid sequences. Residues specific to murine CCR5 are in red. The amino acid changes caused by CCR5 point mutations are indicated above the human CCR5 sequence. (B) peptide N-glycosidase F (PNGase F) digestion of CCR5 coreceptors with ECL2 point mutations. [^{35}S]-Methionine radiolabeled cell extracts from 239T cells transfected with WT CCR5 and CCR5 constructs encoding novel glycosylation sites were immunoprecipitated with the anti-CCR5 MAb 2D7. Immunoprecipitates were subjected to digestion with PNGase F (+) or buffer alone (-) and fractionated by SDS-PAGE. Upper and lower arrows denote glycosylated and deglycosylated proteins, respectively. Mock cells were transfected with the empty pcDNA3.0 vector. (C) HIV-1 infections mediated by ECL2 mutants of CCR5. The coreceptor activity of CCR5s with ECL2 point mutations or a chimeric CCR5 with mouse ECL2 was assessed by using transiently transfected HeLa-CD4 cells as targets for HIV-1_{JR-CSF} infections. Foci of infection were detected using the focal infectivity assay. Relative infectivities were obtained by dividing titers obtained in each transfection condition by cells transfected with wild-type CCR5. The data is a representative experiment of three performed. Error bars are the range.

Supplementary Figure 2. HIV-1 envelope glycoprotein processing and incorporation into virions. Cell extracts and virion pellet fractions were harvested from transfected COS7 cells producing HIV-*gpt* virions pseudotyped with either wild-type or CCR5(HHMH) adapted gp120s. Virion pellets solubilized in sample buffer or envelope glycoproteins immunoprecipitated from cell extracts were resolved by SDS-PAGE and then subjected to western blotting using a sheep anti-gp120 polyclonal antibody. Lane 1: mock (cells were transfected with HIV-*gpt* and an empty pcDNA3.0 vector), lane 2: wild-type

JRCSF, lanes 3, 4, and 5: CCR5(HHMH)-adapted gp120's with the F313L, F313L/N403S, and S298N/F313L/N403S/A428T mutations, respectively, lane 6: SVIIIenv (HXB2 strain).

Supplementary Figure 3. HIV-1 variants that bind CCR5 with the same affinity but require different minimum numbers of associated CCR5s for infection would differ dramatically in their abilities to infect cells. In this analysis, we assumed that the viral fusion complexes contain three binding sites that associate independently with CCR5 with a uniform dissociation constant **k** in accordance with the binomial theorem. The probabilities that the viral complexes would be fusion competent strongly depends on the cellular concentration of CCR5 and on the requisite stoichiometry of the virus. The graph also shows how a redundancy of fusion complexes on the virions would affect the probability that the virus would be fusion competent. Thus, a virus with two complexes that independently associate with CCR5 would infect more efficiently than a virus with only one. This is illustrated for the case where a fusion complex requires at least two associated CCR5s.

SupplementaryFigure 4. Kinetic factors control the efficiencies of HIV-1 infections. The analysis is based on equations S2 and S3. As shown in panels A, the efficiencies **E** of infection depend strongly on the rate constant for membrane fusion $\mathbf{k_i}$ relative to the rate constant $\mathbf{k_d}$ at which virions on HeLa cell surfaces become inactivated or shed. Therefore, HIV-1 is in a competitive race between entry and inactivation, and in this circumstance the efficiency of infection strongly depends on $\mathbf{k_i}$. The left panel shows the effect of the $\mathbf{k_i/k_d}$ ratio on infection of a virus with N=3 binding sites for CCR5 that becomes fusion competent when one or more of these sites is occupied, whereas the right panel shows the effect on a virus that requires at least two associated CCR5s. Panels B show the effects of changing the $\mathbf{k_i/k_d}$

ratio on the normalized $\mathbf{E/E_{max}}$ value of the infection. Clearly, the $\mathbf{E/E_{max}}$ values in panels B are much less affected by changes in $\mathbf{k_i/k_d}$ ratio than the **E** values in panels A. Consistent with equation S5, the $\mathbf{E/E_{max}}$ values become very close to **P** when $\mathbf{k_i/k_d}$ ratios are less than 0.1, which is the case for our experiments. The abscissas in these graphs are **[CCR5]/k**, where **k** is the dissociation constant of the complex.