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Ternary Complex Formation of Human Immunodeficiency Virus Type 1 Env, CD4, and Chemokine Receptor Captured as an Intermediate of Membrane Fusion

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Human immunodeficiency virus (HIV) Env-induced fusion is highly temperature dependent. When effector and target cells were coincubated at 37°C, there was a kinetic delay before fusion commenced. When effector and target cells were coincubated for varied times at 23°C, a temperature that does not permit fusion, a temperature-arrested stage was created. Raising temperature to 37°C from the 23°C intermediate eliminated the kinetic delay. Inhibitors (T22, AMD3100, and Sch-C) that block fusion by binding chemokine receptors were added after creating the intermediate so as to assess the extent of engagement between gp120 and chemokine receptors at that stage. For both CXCR4 and CCR5 as coreceptors, increasingly long times of coincubation at 23°C reduced the efficacy of the coreceptor-binding inhibitors in blocking fusion. This implies that an increasing number of ternary Env/CD4/coreceptor complexes form over time at 23°C. It also shows that ternary complex formation has a lower temperature threshold than the downstream steps that include Env folding into a six-helix bundle; this provides an experimental means to separate coreceptor binding by gp120 from the subsequent refolding of gp41 into a six-helix bundle structure. As the time of cell coincubation at 23°C was prolonged, more cells quickly fused upon the raising of the temperature to 37°C, and the increase quantitatively correlated with the greater percentage of fusion that was resistant to drugs. Therefore the pronounced kinetic delay in HIV Env-induced fusion is caused predominantly by the time needed for ternary complexes to form.

Fusion induced by human immunodeficiency virus (HIV) Env is a multistep process. The gp120 subunit undergoes sequential conformational changes as it interacts with CD4 and its coreceptors (CXCR4 and/or CCR5) (see references 5, 10, and 25 and references therein). The gp120 changes directly lead to the conformational changes of the gp41 subunit that cause fusion between the viral envelope and cell membrane. When Env has engaged both CD4 and its coreceptor, this complex is called a ternary complex (24). The structure of a simplified version of a ternary complex consisting of gp120 with some of its loops deleted, the binding domain of CD4, and a monoclonal antibody that serves as a surrogate for the chemokine receptor have been determined (24). The kinetics of ternary complex formation and its relation to the kinetics of fusion are not known.

Drugs that block fusion by binding to chemokine receptors—thereby preventing Env from engaging its chemokine receptors—are currently in development as therapeutics against HIV infection (10, 25). Because such drugs can be effective only prior to the formation of stable ternary complexes, they can be used to determine at which stage in the fusion process ternary complexes form. Intermediate stages of the fusion process have been captured by coincubating, under conditions suboptimal for fusion, effector (E) cells that express fusion proteins on their surfaces and target (T) cells that express appropriate receptors on their surfaces. For HIV type 1 (HIV-1) Env-induced fusion, an intermediate has been captured by incubating E/T cells together at a temperature (23°C) that is slightly below that needed to induce fusion (13, 16, 32). This state is known as a temperature-arrested stage (TAS).

Fusion between E/T cells induced by HIV-1 Env is a slow, asynchronous process, exhibiting a pronounced lag and taking hours to reach its plateau at 37°C (13, 32). We found that after raising temperature to 37°C from TAS, fusion occurred more quickly and without a pronounced delay. Thus, TAS is a kinetically advanced stage of fusion. Fusion is resistant to inhibitors of CD4 binding at TAS, showing that Env has engaged its receptor at this intermediate (32). In the present paper, we analyze Env-coreceptor interactions at TASs for both X4- and R5-tropic HIV-1 Envs through studies of kinetics and of the potency of fusion inhibition for drugs that bind chemokine receptors. We have found that Env interacts with its coreceptors at TAS. Therefore the creation of TAS ensures the attainment of the ternary complexes without allowing the late refoldings of gp41 that ultimately lead to fusion. We found that from TAS, the kinetic delays that are normally present until fusion have been largely eliminated. This indicates that ternary complex formation is the molecular process responsible for the kinetic lags displayed by HIV Env-induced fusion.

MATERIALS AND METHODS

Cell lines. TF228.1.16 cells stably expressing HIV-1 BH10 (X4) Env were obtained from Z. Jonak (Glaxo SmithKline, Philadelphia, PA) (20) and grown in RPMI 1640 supplemented with 10% fetal calf serum as described previously (32). HeLa cells stably expressing HIV-1 ADA (R5) Env (referred to as HeLa/

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ADA) were a kind gift from M. Alizon (Cochin Institute, Paris, France) (38). A HeLa cell line that constitutively expresses high levels of CD4 and CCR5 (HeLa-JC5.3) (22) was a kind gift of D. Kabat (OHSU, Portland, Oregon). The HeLa/ADA and HeLa-JC5.3 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% penicillin and streptomycin. Cells expressing high levels of ADA Env were periodically selected from the population by adding 2 mM methotrexate. The kinetics and/or extents of fusion generally increase with the densities of fusion protein (29, 33) (our unpublished data). The temperature threshold for fusion consequently decreases for increased density. For high expression levels, some fusion occurred at 23°C. With increased passage, expression levels of ADA Env decreased as evidenced by fluorescence-activated cell sorter analysis (data not shown), the percentage of cells that fused at 37°C decreased, and fusion did not occur at 23°C. Unless otherwise indicated, experiments were performed with these HeLa/ADA cells expressing moderate levels of Env. HeLaT4$^+$ cells expressing CD4 and endogenous levels of CXCR4 were obtained from R. Axel (30) through the NIH Research and Reference Reagent Program and maintained as described previously (32).

Cell labeling and reagents. Effector cells expressing HIV Env were labeled with the green cytoplasmic marker calcein acetoxymethyl (CaAM), and target cells expressing CD4 and coreceptor were labeled with blue CMAC (7-amino-4-chloromethylcoumarin). Labeling was performed according to the manufacturer's instructions. All fluorescent dyes were purchased from Molecular Probes (Eugene, OR). The polyphemusin-derived peptide, T22, was purchased from Bachem Bioscience (King of Prussia, PA) and the bicyclam AMD3100 (7) was obtained through the NIH Research and Reference Reagent Program. A small-molecule inhibitor of CCR5 binding, Sch-C, was kindly provided by J. Strizki (Schering-Plough Research Institute, New Jersey) (40). The anti-CCR5 monoclonal antibody PA14 (34) was a kind gift of W. Olson (Progenics Pharmaceuticals, Tarrytown, NY). C34 peptide derived from the C-terminal heptad repeat domain of gp41 (HXB2 strain) was synthesized by Macromolecular Resources (Fort Collins, CO).

Fusion experiments and creating TAS. Fusion between E and T cells was carried out and measured as described previously (32). Briefly, effector cells loaded with CaAM were mixed with target cells labeled with CMAC and incubated at 37°C for indicated periods of time. The kinetics of fusion was measured by stopping cell-cell fusion at different times after starting the coincubation. Fusion was stopped by the addition of 0.5 μM C34 peptide to the cell mixture, a concentration more than 10-fold greater than the 90% inhibitory concentration of X4 and more than 5-fold greater than the 90% inhibitory concentration of R5 Env. The percentage of fusion is defined as the percentage of E/T cell pairs in contact that exhibited CaAM and CMAC mixing. When considering the efficacy of a drug, the percentage of fusion in the absence of the drug is set as 100% (see Fig. 2). We operationally defined the lag or delay time until fusion as the minimum time that we had to maintain bound cells at 37°C before some fusion could be detected. If, after creating an intermediate, we observed fusion at our earliest observation time after raising the temperature to 37°C, then by our definition there was no delay time prior to fusion. To create TAS, E/T cells were cocultured for various times (2.5 h unless indicated otherwise) at 23°C ± 1°C within a temperature-controlled incubator. We previously created TAS by coincubating the cells at room temperature, nominally ~23°C (32). Because the intermediate stage reached is not as advanced for temperatures slightly less than 23°C (see Fig. 4A), the TAS intermediate of the present study may not be exactly the same as that of our previous study (32). The kinetics of cells fusing from TAS within 5 min (i.e., fast-fusion kinetics) after a temperature rise to 37°C was measured by combining real-time video microscopy and a temperature-jump protocol (32). Inhibitory agents were added either at the beginning of cell coincubation at 37°C or at TAS. When added at TAS, the agents were allowed to bind for 10 min at room temperature, and E/T cells were warmed to 37°C (for 30 to 60 min as indicated below, e.g., see Fig. 2). The kinetics of fusion from TAS and the ability of inhibitors directed against chemokine receptors (e.g., AMD3100 or Sch-C) to block fusion when added at TAS were quantitatively quite reproducible, not varying over the multiple cell passages made over the course of weeks. This is reflected by relatively small error bars (see Fig. 2 and 3). There was, however, quantitative variability when comparing inhibitions of cells that were used months apart. We therefore limited all comparative studies to cells that were used only several weeks apart. But even for cells used months apart, all effects we report were qualitatively reproducible. Most importantly, the shorter the delay in the commencement of fusion when immediately incubating E/T cells at 37°C, the less effective were the drugs in inhibiting fusion.

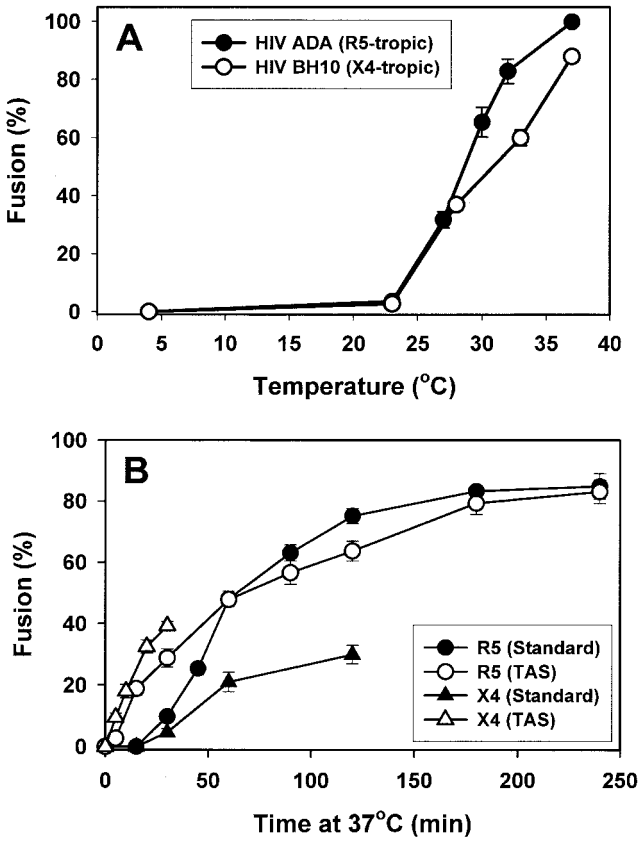

FIG. 1. (A) Temperature dependence of fusion between BH10 (X4) Env-expressing cells and HeLaT4$^+$ cells (open circles) and between ADA (R5) Env-expressing and HeLa-JC5.3 cells (filled circles). E/T cells were incubated at the indicated temperatures for 3 h and fusion was measured. In the case of ADA Env-induced fusion at 37°C, however, cells were incubated for only 1 h; for longer incubations, syncytia formed, hindering quantification. The extent of fusion at a given temperature was expressed as the percent obtained at 37°C. (B) Kinetics of fusion between ADA Env-expressing and HeLa-JC5.3 cells (filled circles) and between TF228.1.16 (X4) and HeLa T4$^+$ cells (filled triangles) in a standard 37°C protocol. For fusion from TAS, R5 Env-expressing (open circles) and X4 Env-expressing (open triangles) cells were preincubated with their target cells at 23°C for 2.5 h prior to a temperature rise to 37°C. Data points are the averages of at least three independent measurements (± standard errors).

RESULTS

The temperature dependences of fusion are similar for X4- and R5-tropic HIV-1 Env. We and others have previously shown that X4-Env induces E/T cell fusion at temperatures above 23°C (2, 11, 32). In the present study, we measured the temperature dependence of fusion induced by an R5-Env (ADA) and compared it to fusion induced by X4-Env. Effector cells expressing X4- or R5-Env were incubated with CD4$^+$ target cells that expressed the appropriate chemokine receptors. Fusion induced by HIV-1 Env was a steep function of temperature and was independent of coreceptor tropism (Fig. 1A). The temperature threshold, ~23°C, was also relatively independent of tropism. We measured the temperature threshold for several strains of X4-Envs (BH10 of this study, HXB2, and codon-optimized MN) and found that they all shared the common threshold of ~23°C (data not shown). However, the

threshold did vary somewhat with R5-Env expression density: for the highest density of ADA Env we achieved, there was a small but measurable amount of fusion once E/T cells were coincubated for 3 h at 23°C (data not shown); at temperatures of ≤20°C, fusion did not occur, a result consistent with the threshold previously found for other CCR5-tropic strains (1, 19). For this study, we used cells that expressed levels of ADA Env somewhat below the maximum that could be achieved, so that fusion did not occur at 23°C. We could thus create TAS at 23°C for both X4- and R5-Env, facilitating comparison of the intermediates for the two tropisms.

TAS is a kinetically advanced intermediate of fusion for both X4- and R5-tropic Env. Raising temperature to 37°C from TAS for an X4-tropic (Fig. 1B) is known to yield a much faster rate of fusion than that which occurs by coincubating E/T cells at 37°C from the start (referred to as the standard protocol [32]). We have now found that R5 Env-induced fusion is also kinetically advanced at TAS (Fig. 1B). We created this R5-tropic TAS by preincubating E/T cells for 2.5 h at 23°C and compared its kinetics of fusion against that obtained for the standard protocol (Fig. 1B). The most prominent difference is that the lag time until fusion was virtually eliminated by creating the intermediate, although the speed-up in fusion was less pronounced than that for the X4-Env. Also, only about half of the population was kinetically advanced at TAS for R5-Env. Thus, for both X4- and R5-Env, a population of Env overcomes multiple slow steps at a subthreshold temperature; these steps delay fusion in the standard protocol. For those cell pairs that were advanced at TAS, fusion was relatively synchronized from the same starting point. Having been subjected to a suboptimal temperature, the kinetically advanced cells have completed their early, upstream steps, and this allows for study of these steps and of the captured TAS intermediate without interference from the later, more highly temperature-sensitive steps required for fusion.

Both X4- and R5-Env form ternary complexes with CD4 and chemokine receptors at TAS. We previously found that when added at TAS, reagents that prevent CD4 from binding Env did not inhibit fusion, indicating that Env had fully engaged CD4 (32). In the present study, we investigated whether X4-Env had engaged CXCR4 by measuring the dose dependence of the inhibition of fusion by T22 and AMD3100 added at TAS. Both reagents bind to a region (i.e., an epitope) on CXCR4 that must interact with Env for the trimeric complex to form. If Env has not yet engaged the epitope, the drugs block fusion. Even though low concentrations of the inhibitors blocked all fusion when added at the beginning of coincubation at 37°C (i.e., the standard protocol) (Fig. 2A and B), only a fraction of the fusion events were inhibited, no matter how much AMD3100 was added at TAS. The longer the time E/T cells were preincubated at 23°C before the addition of AMD3100, the greater was the extent of fusion upon raising the temperature to 37°C (Fig. 2A). After a 2.5- to 3-h preincubation at 23°C, the highest concentrations of either AMD3100 or T22 inhibited only ~40% of the fusion events. The reduced ability of the reagents to inhibit fusion as the time of coincubation at a subthreshold temperature is lengthened implies either that more copies of Env engaged CXCR4 or that engagement between individual proteins became tighter. Our experiments thus revealed that Env and CXCR4 have engaged

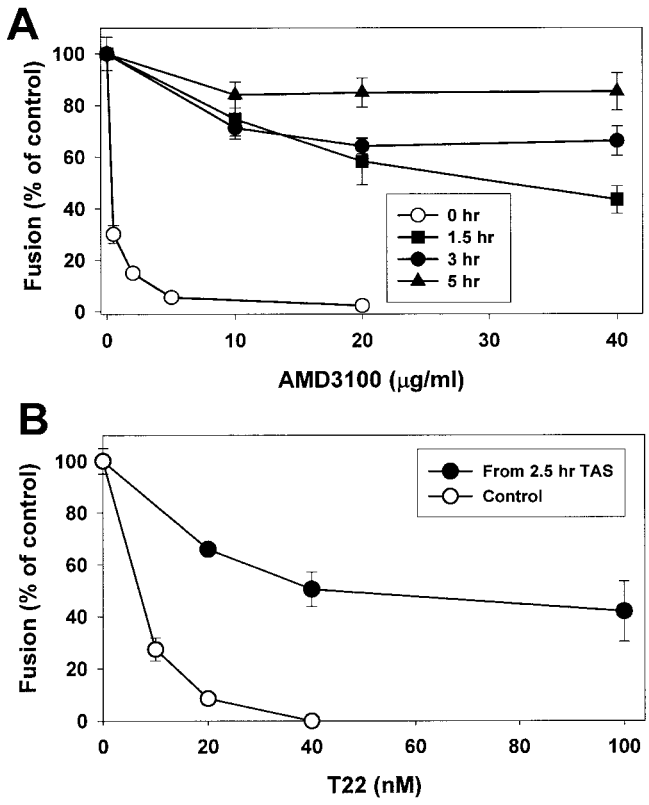

FIG. 2. Inhibition of fusion between BH10 Env-expressing and HeLaT4$^+$ cells by AMD3100 (A) and T22 (B). (A) AMD3100 was added to E/T cells either at the beginning of a standard 37°C incubation (open circles) or after preincubation at 23°C for 1.5 h (filled squares), 3 h (filled circles), or 5 h (filled triangles). (B) Inhibition of fusion by T22 peptide added at the beginning of cell coincubation (open circles) or after 2.5 h at TAS (filled circles). When AMD3100 or T22 was added at TAS, the inhibitors were allowed to bind to cells for 10 min at 23°C, and cells were warmed to 37°C for 1 h.

at TAS. Our findings contrast with those of a prior study which found that AMD3100 inhibited all fusion when added at TAS (13).

We investigated whether R5-Env had also bound its chemokine receptors by the point of TAS. We assessed the degree of gp120 engagement to CCR5 by measuring the efficacy of Sch-C in inhibiting fusion when added after preincubating E/T cells at 23°C for various times. Binding of Sch-C to CCR5 blocks fusion, probably by causing allosteric changes that prevent the chemokine receptor from engaging HIV Env (4, 9, 42). Sch-C potently inhibited cell fusion when added at the beginning of the standard protocol, maintaining 37°C for 1 h (Fig. 3A). However, fusion was only partially blocked when the drug was added after coincubating cells at 23°C. As was the case for the inhibition of X4-Env-induced fusion by AMD3100, the shapes of the dose-response curves of inhibition clearly showed that there were two distinct populations of cell pairs after the coincubations at 23°C: fusion was potently inhibited by Sch-C for one fraction but not inhibited at all for the other. The greater the time of coincubation, the greater was the fraction of cells that fused, despite the addition of Sch-C; virtually the full extent of fusion occurred upon raising the temperature to 37°C

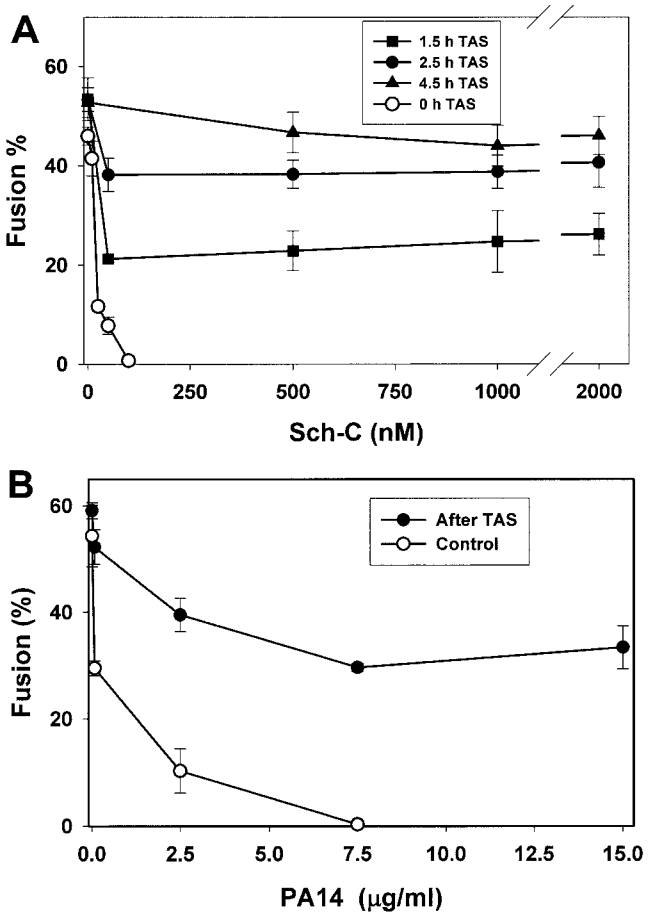

FIG. 3. Inhibition of fusion between ADA Env-expressing and JC-5.3 cells by Sch-C (A) and the anti-CCR5 antibody PA14 (B). For panel A, Sch-C was added either at the beginning of E/T cell incubation at 37°C or after E/T cells were preincubated at 23°C for 1.5 h, 2.5 h, or 4.5 h. Sch-C was present for 10 min at 23°C, followed by a 1-h incubation at 37°C. Panel B shows that when introduced at TAS, PA14 antibody was allowed to bind to cells for 30 min at 23°C before the temperature was shifted to 37°C.

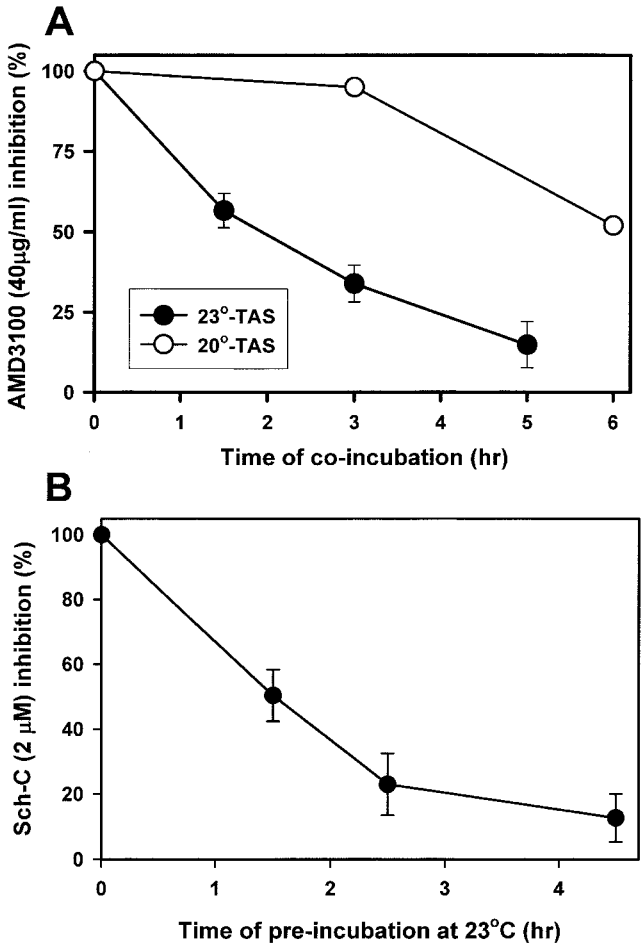

FIG. 4. The time courses for increased resistance of fusion against chemokine receptor-binding drugs. Inhibition of fusion by 40 μg/ml AMD3100 (filled circles) (A) or by 2 μM Sch-C (B) as a function of time of preincubation at 23°C was plotted from data in Fig. 2 and 3. Fusion inhibition by AMD3100 added after various times of preincubation at 20°C is shown by open circles in panel A. Sch-C was more potent in inhibiting fusion after a preincubation at 20°C rather than one at 23°C (panel B), but the extents of inhibition after various times of 20°C preincubation were not quantitatively determined.

after a 4.5-h coincubation at 23°C, no matter how much Sch-C was added to the intermediate (Fig. 3A). In short, the Sch-C inhibition data indicate that gp120 can engage CCR5 at 23°C and that the engagement increases over a time course of hours. We interpret the increased engagement as being due to the formation of more ternary complexes, but we cannot rule out the possibility that it is due to Env and CCR5 contacting each other over more of their binding sites, reducing the effectiveness of Sch-C. Fusion was also inhibited by adding the neutralizing mAb PA14, which blocks fusion by competing with gp120 for sites on the N terminus and sites on the second extracellular loop (ECL2) of CCR5 (34). PA14 completely inhibited fusion when added at the start of E/T cell coincubation at 37°C (Fig. 3B). After incubating E/T cells together for 2.5 h, however, the addition of the antibody could block only ~50% of the possible fusion events (Fig. 3B), which is consistent with the incomplete inhibition of fusion by Sch-C. Thus, ternary complexes between Env, CD4, and CCR5 form at 23°C.

The increased protection against the inhibition of fusion by AMD3100 for X4-tropic Env and against Sch-C for R5-tropic Env as the incubation time of E/T cells at 23°C is prolonged provides a means to evaluate the kinetics of CXCR4 and CCR5 engagement with Env. That is, the loss of potency of a drug corresponds to the formation of ternary complexes. We used concentrations of AMD3100 (40 μg/ml) (Fig. 4A) and Sch-C (2 μM) (Fig. 4B) that were much higher than would be needed to maximally inhibit fusion in the standard protocol. The time courses for increased protection against the drugs were lengthy, and both X4- and R5-tropic Env exhibited the same half-times of ~1.5 to 2 h.

Ternary complex formation is highly temperature dependent. At 37°C, fusion begins after a lag of ~20 min, whereas at 23°C, Env-coreceptor binding is rather slow and fusion does not occur. We therefore tested whether Env and coreceptors would associate at a temperature lower than 23°C. We incu-

bated E/T cells at 20°C and found that, after 2.5 h, the addition of AMD3100 inhibited all fusion when the temperature was raised to 37°C for 1 h (Fig. 4A). However, adding the inhibitor after a 6-h coincubation at 20°C led to only ~50% of the maximum number of fusion events. Thus, Env does engage CXCR4 at 20°C, but the engagement is much slower than at 23°C. After coincubating cells for several hours at temperatures below 20°C, we observed neither speed-up in fusion kinetics nor protection against AMD3100. Therefore, the phenomenon of ternary complex formation can be studied only over a limited temperature range. From the rate of complex formation at 20°C and at 23°C, the extrapolated increment in the rate of ternary complex formation for a 10°C increase in temperature (the Q_{10}) is ~10.

If E/T cells become resistant to the inhibitors at TAS because of ternary complex formation, as we have concluded, then these cell pairs should fuse more quickly upon a temperature rise to 37°C than do the cell pairs still susceptible to the inhibitors. If this conclusion is correct, then more cell pairs should quickly fuse as the time of coincubation at 23°C is increased, but their fusion kinetics will be independent of the coincubation time. We tested the validity of our conclusion by measuring the extent and kinetics of X4-tropic cell pairs that quickly (within 5 min) fused at 37°C after various times of preincubation at 23°C. The extents of fusion were proportional to the preincubation times (Fig. 5A), and the kinetics of fusion (after renormalizing for differences in extents) (Fig. 5A, inset) were insensitive to the preincubation times, in support of our conclusion. Further support is provided by the finding that the increase in the number of cells that quickly fused after greater times of coincubation at 23°C (normalized by the number that fused after a 5-h coincubation) (Fig. 5B) qualitatively matched the fraction that could no longer be blocked by AMD3100 after long times of coincubation (Fig. 4A). The fact that more ternary complexes continued to form at 23°C but fusion still did not occur underscores the finding that a higher temperature is needed for pore formation than for ternary complex formation.

Coreceptor-binding inhibitors do not block fusion once ternary complexes have formed. To explore the consequences of the allosteric changes CCR5 undergoes after binding Sch-C, we analyzed the kinetics of fusion in the presence of different concentrations of Sch-C. In the standard protocol, the lag time was greater in the presence of a concentration (50 nM) of Sch-C that inhibited about half of the fusion events than it was in the absence of Sch-C (Fig. 6A). However, once fusion started, the kinetics in the presence of 50 nM Sch-C was not significantly different from that of the standard protocol. A higher concentration of Sch-C (250 nM) further reduced the extent of fusion (Fig. 6A) and clearly slowed the kinetics, but it did not further increase the lag time. Because 250 nM Sch-C is ~25 times greater than its dissociation constant from CCR5 (K_d = 9.3 nM [40]), the drug should have bound virtually all copies of CCR5. However, this concentration of Sch-C did not completely block fusion, indicating either that Env can bind CCR5 while Sch-C is bound and still participate in fusion (albeit with less effectiveness) or, as we consider more likely, that Env engages CCR5 while the binding site of Sch-C is transiently vacated (23). For this latter explanation to be possible, the binding of Sch-C would have to be reversible, but this would be contrary to a prior report (43). We therefore tested whether the inhibition of fusion by Sch-C was eliminated by a washout and found that it was (Fig. 6A, inset). The inhibition of fusion by Sch-C is therefore reversible.

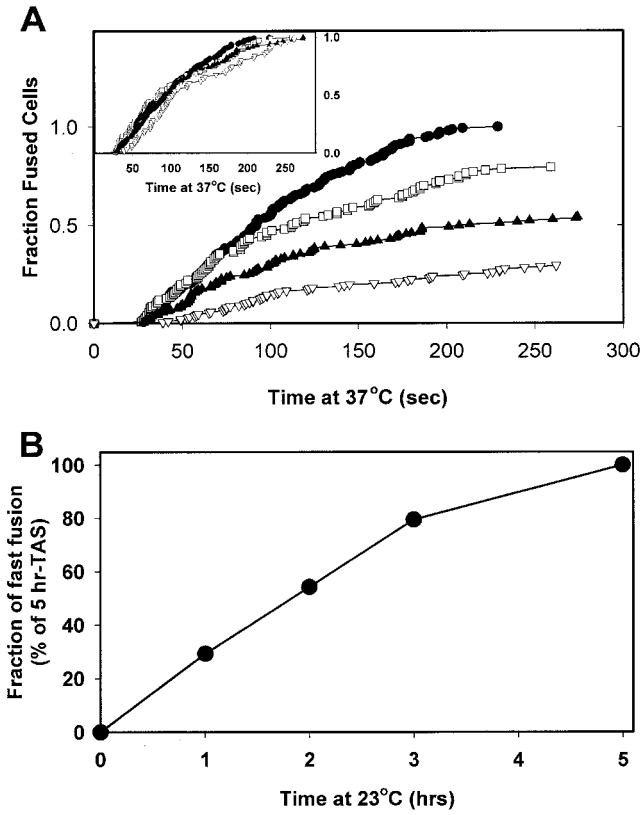

FIG. 5. (A) Kinetics of fusion between BH10 Env-expressing and HeLaT4$^+$ cells induced by quickly stepping temperature to 37°C after preincubations at 23°C for 1 h (open inverted triangles), 2 h (filled triangles), 3 h (open squares), and 5 h (filled circles). Cells were exposed to 37°C for 5 min, and fusion between individual cell pairs was monitored by video microscopy. The extents of fusion after various times of preincubation were normalized to that after the 5-h preincubation. (Inset) Each curve is renormalized to have the same extent of fusion. The kinetic curves almost superimpose, demonstrating equivalent kinetics. (B) Fraction of E/T cells in contact that fused after a 5-min 37°C exposure as a function of preincubation time at 23°C.

We analyzed the effect on the kinetics of fusion when Sch-C is added at the point of TAS. Fusion did not exhibit an appreciable lag time from TAS, without regard to whether the drug was added at a high concentration, a partially inhibiting concentration (Fig. 6B), or not at all (Fig 6B and 1B). The drug reduced the final extent of fusion without appreciably affecting kinetics. The simplest interpretation is that ternary complexes had already formed for those cells that quickly fused from TAS upon a temperature rise to 37°C; the addition of Sch-C affected only CCR5 that had not already bound Env. Sch-C either did not bind CCR5 that was part of the ternary complex or, if it did (23), the binding did not prevent fusion. It is noteworthy that when adding 250 mM Sch-C at TAS, the extent of fusion showed a clear plateau after 1 h of raising the temperature to 37°C (Fig. 6B). The most likely explanation is that the high concentration of Sch-C prevented the additional recruitment

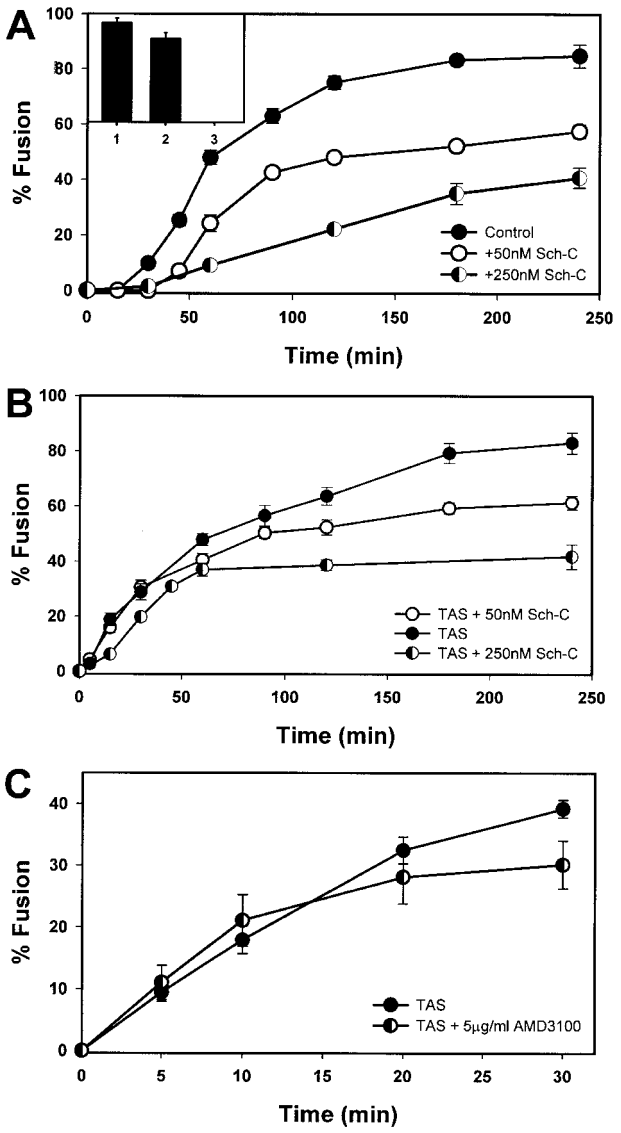

FIG. 6. Effect of Sch-C on the kinetics of fusion between ADA Env-expressing and HeLa-JC5.3 cells. (A) Cells were directly incubated at 37°C, and Sch-C was added at concentrations of 50 nM (open circles) or 250 nM (semifilled circles) or was not added (filled circles). The cells used for this set of experiments were not the same as those for Fig. 3A; the experiments for Fig. 6 were performed many months after those for Fig. 3A. Fluorescence-activated cell sorter analysis showed that the expression levels of Env were greater for those for these latter cells, accounting for the somewhat reduced ability of Sch-C to inhibit fusion. Whereas 250 nM inhibited all fusion after 1 h at 37°C for the experiments of Fig. 3A, here 5 to 10% of the cells fused over this time in the presence of the drug. (Inset) The target cells were incubated with 500 nM Sch-C for 15 min at room temperature. The drug was either washed out (column 2) or maintained (column 3); the target cells were mixed with effector cells for 1 h at 37°C, and fusion was measured. In control experiments, the drug was never added (column 1). Bar 1 is set to 100%. (B) TAS was created (2.5 h at 23°C), and 50 nM (open circles) or 250 nM (semifilled circles) Sch-C was allowed to bind for 10 min at room temperature (filled circles, drug not added), and fusion was then triggered by raising the temperature to 37°C for 1 h. Because fusion is observed at the first time point of observation (5 min), a kinetic delay is absent according to our definition. (C) Kinetics of fusion between TF228.1.16 cells and HeLaT4$^+$ cells were measured from TAS without adding (filled circles) or after adding (semifilled circles) 5 μg/ml AMD3100 for 10 min before raising the temperature to 37°C. The kinetics for the standard protocol are shown in Fig. 1B.

of CCR5 into ternary complexes. The kinetics of fusion subsequent to ternary complex formation would thus be the same as that which occurs subsequent to the addition at TAS of a high concentration of a blocking reagent.

We performed additional experiments to further demonstrate that for X4-Env, the kinetics of post-TAS fast fusion is the same as the kinetics of fusion subsequent to ternary complex formation. We created TAS for X4-Env and compared the kinetics of fusion in the absence and the presence of AMD3100 (5 μg/ml). The addition of AMD3100 did not affect the kinetics of fusion from TAS (Fig. 6C). In other words, for both X4- and R5-Env, once TAS is created, small-molecule drugs that bind the chemokine receptor do not affect the kinetics of fusion. Collectively, our results indicate that molecules that inhibit fusion by binding to coreceptors are without effect once ternary complexes have formed. Resistance to the drug is independent of the Env-coreceptor affinity and independent of the drug mechanism. We conclude that ternary complexes reach a stage at which they are stable, and at that point, kinetic lags have been eliminated. That is, the time it takes for ternary complexes to form is the dominant cause of the kinetic lags. Fusion is still relatively slow, however, occurring over a time course of tens of minutes. One or more kinetic barriers must still be overcome to create a fusion pore at the physiological temperature of 37°C.

DISCUSSION

We captured an intermediate of HIV-1 Env-induced fusion for X4-tropic Env, and one for R5-tropic Env, both through the use of a subthreshold temperature. For both tropisms, kinetic delays of fusion were eliminated at TAS. Based on the abilities of drugs that prevent fusion by binding chemokine receptors, we conclude that, independently of tropism, Env engages coreceptors at the intermediate stage of a 23°C TAS (Fig. 7). TASs are essentially the same for both tropisms. It is striking that ternary complex formation, fusion pore creation, and pore enlargement—sequential steps of HIV Env-induced fusion—exhibit different temperature thresholds (Fig. 7): pore creation requires the highest temperature, and pore enlargement permits the lowest (~15°C [31]). Our finding that the formation of ternary Env/CD4/coreceptor complexes can occur at a lower temperature than pore opening provides a means to isolate the formation of ternary complexes from the subsequent, downstream steps that lead to pore formation. The isolation of ternary complex formation could potentially be utilized in either drug development or mechanistic studies of fusion. For example, after creating an intermediate stage at which ternary complex formation has been completed (e.g., TAS), fusion will have been kinetically synchronized, so that late, downstream steps can be characterized without interference from prior and slower steps. The procedures and results of the present study should generalize to and be applicable to the biological process of fusion between viruses and cells. In fact, we have found that by binding HIV Env-pseudotyped virions to cells at a suboptimal temperature, a temperature-arrested stage is created such that fusion occurs rapidly and synchronously upon a temperature rise to 37°C (R. M. Markosyan, F. S. Cohen, and G. B. Melikyan, submitted for publication).

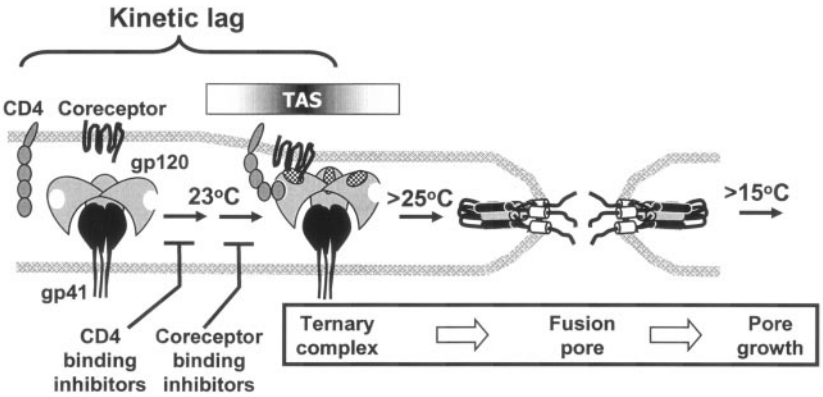

FIG. 7. Temperature requirements for sequential steps of fusion. Ternary complex formation can occur at a lower temperature than creation of the pore can. Drugs that bind chemokine receptors are active only prior to the formation of stable ternary complexes. The time of ternary complex formation determines the kinetic lag.

The absence of a kinetic delay for fusion induced from TAS indicates that the delay that occurs in the standard protocol is caused by a combination of steps that lead to ternary Env/CD4/coreceptor complexes. Such steps would include the large-scale conformational changes gp120 subunits undergo after they bind CD4 (5). By adding, at TAS, a fusion inhibitor that binds coreceptor, those cells with completed complex formation can be functionally differentiated from the rest of the cell pairs. When the temperature is raised to 37°C after adding the inhibitors, fusion occurs within 1 h (Fig. 6B); there is no lag, and fusion is faster and is completed for all cells sooner than in the standard protocol (Fig. 6A). Despite the kinetic advances, fusion is still relatively slow after ternary complex formation. One reason fusion could still be spread out over a long time is that the cells are not homogeneous—they have different densities of Env and coreceptors and all ternary complexes may not have identical stoichiometries. Slow fusion from TAS can also be explained mechanistically, based on the high level of temperature dependence of the process of fusion proceeding from TAS. The steep temperature dependence could, in principle, be due to the cooperative change of several Envs, each with a relatively small energy barrier against conformational transitions. However, HIV contains few copies of Env (6, 45), and therefore not many copies could participate in fusion (44). We consider it likely that the steep temperature dependence arises from a high energy barrier against a conformational change of an individual Env. The difficulty of gp120's separation from gp41 could be the cause of such a barrier. When a disulfide bond is engineered between gp120 and gp41 subunits, this SOS-Env can engage its chemokine receptor with the engineered SS bond intact. Reduction of the SS bond at 23°C, after engagement, does not yield fusion; fusion requires that the temperature be raised (1). This suggests that once ternary complexes have formed, the separation of gp120 from gp41 is the most difficult energetic step. Once maximal separation occurs, gp41 spontaneously reconfigures from a metastable state into a six-helix bundle configuration. Alternatively, the barrier could arise because of steric hindrances during the reconfiguration of gp41 into a six-helix bundle. Any process that occurs prior to pore formation contributes to the kinetic delay. For example, an association between multiple copies of Env likely occurs after ternary complex formation. This might be thought to contribute significantly to the kinetic lag. However, our data strongly indicate that the kinetic lag is negligible once a ternary complex has formed and thus that such Env-associations should not be of consequence for the kinetic delay.

HIV-1 Env binds to multiple epitopes that have been mapped to the N-terminal domain and to extracellular loops of a chemokine receptor (14, 27, 35). The small-molecule inhibitor Sch-C blocks R5-tropic fusion by binding to a hydrophobic pocket formed by TM helices 1, 2, 3, and 7 of CCR5 (9, 42). Sch-C binding is thought to cause an allosteric conformational change in CCR5 (4, 42) which prevents the CCR5 from engaging gp120. The difficulties in interpreting the inhibition caused by allosteric conformational changes are illustrated by the finding that a point mutation introduced into human CCR5 rendered Sch-C ineffective, even though the drug probably bound to the mutant (4). The precise binding sites for CXCR4 antagonists are not fully identified. However, recent mutagenesis and modeling studies suggest that the structurally unrelated T22 and AMD3100 fit into the same ligand-binding pocket of CXCR4 (3, 15, 17, 26, 39, 41). Some of the amino acid residues comprising the binding sites are probably the same for the two drugs, including acidic and aromatic residues near the membrane-water interface. Because gp120 probably binds to extracellular domains of the coreceptor that protrude from the membrane, the binding of either T22 or AMD3100 may prevent fusion by causing allosteric changes in CXCR4. If binding of Sch-C, AMD3100, and T22 inhibit fusion by causing allosteric changes and if their binding sites are still exposed at TAS, then Env probably locks the coreceptor into a state that supports fusion independently of drug binding.

The finding that the time courses were the same for both chemokine receptors is surprising: the affinity of the monomeric gp120 of R5-tropic Env to its chemokine receptor is generally much higher than that of X4-tropic Env (8, 18), and fusion kinetics are generally faster for R5-tropic Env (2). Thus, the BH10 Env stably expressed in our effector cells likely binds CXCR4 with lower affinity than our Env ADA binds CCR5. Also, our X4 target cells should express low densities of CXCR4, on the order of 10^3 to 10^4 molecules per cell, which is

the typical range for endogenous expression (28). In contrast, our target cells for ADA Env were selected for their high expression levels of CCR5, which were $\sim 10^5$ per cell (37). Although all these factors should favor Env-CCR5 associations over those of Env-CXCR4, X4-tropic fusion gained protection against AMD3100 and R5-tropic fusion gained protection against Sch-C at about the same rate at 23°C (Fig. 4). The temperature dependencies of fusion were also similar for R5- and X4-tropic Envs (Fig. 1A). Even though monomeric R5-tropic gp120 has an affinity for its chemokine receptor higher than that of X4-tropic forms, the order of affinity is just the opposite for CD4 (21, 36, 37): the higher CD4 affinity of X4-tropic Env would tend to compensate for the higher CCR5 densities and chemokine receptor affinities of tropic Env. The compensation would be greater if more-avid binding of CD4 (at 23°C) led to a more optimal exposure of the regions of gp120 that engage chemokine receptors. In fact, the binding of sCD4 to X4-tropic Env readily exposes the grooves of the trimeric coiled coil of gp41, whereas this exposure does not occur with some strains of R5-tropic Env (12). All in all, one would expect that more CCR5 would be readily recruited into ternary complexes than would CXCR4, when in fact we observe equal rates of protection. It may be that ternary complex formation is not the same for X4- and R5-tropic Env at TAS but that the differences in complex formation are obscured by the different manners in which AMD3100 and Sch-C inhibit fusion. An alternative explanation could be that the rate of trimeric Env binding to its chemokine receptor is more dependent on steric and configurational arrangements than on the affinity of monomeric gp120 for its chemokine receptor. Whatever the cause, it appears that matching the threshold temperature for fusion to 23°C for both X4- and R5-Env resulted in matched kinetics of engagement of Env for its coreceptor.

Based on the temperature dependence for obtaining protection against inhibition of fusion by AMD3100 at 20°C and 23°C, the Q_{10} for the rate of ternary complex formation is ~ 10. This high value is consistent with the conclusion that more than one copy of CXCR4 interacts with an Env in a cooperative process (22). Up to three CXCR4 molecules could interact with the threefold symmetric Env, and it is possible that a multiplicity of engagement is necessary for Env to induce formation and growth of a fusion pore. For Q_{10} of ~ 10, ternary complexes that form over several hours at 23°C (Fig. 4) should form over a time course of ~ 20 min at 37°C. This matches the times of the kinetics delays until fusion. All of our data are consistent with the conclusion that the lag time until fusion is caused by the time it takes to form ternary complexes.

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