Exposure of the Membrane-Proximal External Region of HIV-1 gp41 in the Course of HIV-1 Envelope Glycoprotein-Mediated Fusion†

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ABSTRACT: The membrane-proximal external region (MPER) of HIV-1 gp41 is highly conserved and critical for the fusogenic ability of the virus. However, little is known about the activity of this region in the context of viral fusion. In this study we investigate the temporal exposure of MPER during the course of HIV-1 Env-mediated fusion. We employed the broadly neutralizing monoclonal antibodies 2F5 and 4E10, whose epitopes localize to this region as indicators for accessibility to this region. Time of addition experiments indicated that escape of HIV-1 infection inhibition by 2F5 and 4E10 occurred concomitantly with that of C34, a peptide that blocks the six-helix bundle formation and fusion, which was about 20 min later than escape of inhibition by the mAb b12 that blocks CD4-gp120 attachment. We also probed accessibility of the MPER region on fusion intermediates by measuring the binding of the monoclonal antibodies at different time points during the fusion reaction. Immunofluorescence and in-cell Western assays showed that binding of 2F5 and 4E10 decreased upon triggering HIV-1 Env-expressing cells with appropriate target cells. Addition of C34 did not counteract the loss of antibody binding, suggesting that changes in exposure of MPER occur independently of six-helix bundle formation.

The interactions of HIV-1 Env (gp120-gp41) with CD4 and coreceptors trigger a barrage of conformational changes in Env that drive the membrane fusion process (*1*). An outcome of the reaction is the formation of a six-helix bundled gp41 ectodomain core structure consisting of three N-helical regions (N-HR)[1] paired with three antiparallel C-helical regions (C-HR) (*2*−*5*). HIV fusion can be inhibited by peptides that mimic the sequences of N-HR or C-HR by binding to gp41 C-HR or N-HR, respectively, thereby preventing six-helix bundle formation (*6*−*14*). Although the majority of the attention has been devoted to the role N-HR, C-HR, and six-helix bundle formation play in the fusion reaction, other regions appear to be of equal importance. In this study we focus on the tryptophan-rich region immediately adjacent to the membrane-spanning domain (residues 666−682 of the HXB2 strain of HIV-1), termed the membrane-proximal external region (MPER), which is crucial for the proper functioning of gp41 as a fusion protein (*15*). This region is conserved in the vast majority of otherwise highly variable HIV-1 isolates. Deletion of the entire stretch of 17 amino acids abrogated the ability of the envelope glycoprotein to mediate both cell−cell fusion and virus entry without affecting the normal maturation, transport, or CD4-binding ability. Alanine substitution of the five conserved tryptophan residues produces a phenotype, in which the Env does not induce syncytia but does permit redistribution of small aqueous dyes between host and target cells, indicating a failure in fusion−pore expansion (*16*). To further elucidate the role of MPER in the fusion mechanism, we monitored the kinetics of its exposure after triggering with target cells bearing CD4 and coreceptor.

MATERIALS AND METHODS

Time of addition studies were performed with intact NL3-4 HIV-1 using the TZM-bl indicator cell line (*17*), (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.), which produces luciferase activity as a result of HIV-1 infection. TZM-bl cells were infected with NL4-3 HIV-1 at 37 °C and a MOI of 0.01. The mAbs IgG-b12 (a kind gift from Dennis Burton) (*18*), which blocks gp120-CD4 binding, 2F5 and 4E10, which interact with MPER, or the peptide C34, which blocks six-helix bundle formation, were added at different time points, from 0 to 120 min, postinfection. Luciferase activity was quantified after 16 h incubation at 37 °C, and viral infection was determined relative to the control untreated cells. The data were fit by the standard sigmoidal equation $f(t) = a/(1 + e^{-b(t-t_{1/2})})$ using Sigmaplot software (SPSS, Inc., Chicago, IL).

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; MPER, membrane-proximal external region or membrane-proximal domain; C-HR, C-terminal helical region; N-HR, N-terminal helical region.

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Binding of mAbs to HIV-1 Env in the course of the fusion reaction was performed using Chinese hamster ovary cells stably expressing IIIB HIV-1 Env (CHO-WT) (*19*) and SupT1 cells as targets, obtained through the AIDS Research and Reference Reagent Program from Carol Weiss and Judith White and from James Hoxie, respectively. In the immunofluorescence studies the Env-expressing and target cells were cocultured at 37 °C, and at various time points, between 5 and 30 min, the cells were immediately placed on ice to stop the fusion reaction. The cells were extensively washed, and mAb binding was assessed by quantitative immunofluorescence and fusion by redistribution of calcein from SupT1 cells to Env-expressing cells as described previously (*20*, *21*). In the in-cell Western studies CHO-WT cells were incubated at 37 °C with or without SupT1 cells for 30 and 60 min in the presence or absence of 10 nM C34 and examined for 2F5, 4E10 of D49 mAb binding using the Odyssey infrared detection system (LI-COR Biosciences, Lincoln, NE). The D49 mAb, a kind gift from Pat Earl (*22*), was used as a control to account for unchanged surface expression of HIV-1 Env during the incubation period. After coculture, the cells were washed and fixed with 4% formaldehyde and blocked for 90 min at room temperature with Odyssey blocking buffer. The cells were then incubated with the appropriate primary antibody for 2 h at room temperature in LI-COR blocking buffer containing 0.2% Tween-20, washed five times for 5 min each with 0.1% Tween-20 in PBS (PBST), incubated with 1:800 IRDye 800CW conjugated affinity-purifed anti-human IgG (H&L) (Rockland Immunochemical, Gilbertsville, PA) in LI-COR blocking buffer with 0.2% Tween-20, and washed five times for 5 min with PBST. The signal was normalized to the 700 channel using 1:10000 SYTO red fluorescent nucleic acid stain (Invitrogen, Carlsbad, CA) to control for differences in cell number. Signals from duplicate experiments using nonenvelope expressing cells were subtracted to control for nonspecific antibody binding.

RESULTS

To assess exposure of MPER in the course of the fusion reaction, we examined at which point in the fusion cascade the mAbs 2F5 or 4E10 can neutralize HIV-1. The inhibition kinetics mediated by these two mAbs were compared with those of the mAb b12, which blocks gp120-CD4 attachment (early step in the fusion cascade), and of the peptide C34, which blocks six-helix bundle formation (late step in the fusion cascade). Figure 1 shows the relative infection of TZM-bl cells by NL4-3 HIV-1 upon addition of the mAbs or C34 at different time points between 0 and 120 min. After 60 min of incubation of virus and cells with b12 there was nearly 100% infection, indicating that all of the Envs had escaped inhibition by this mAb by attaching to target cell CD4 molecules. However, at this time point about 50% of those Envs were still available for fusion; i.e., their gp41 molecules had not yet reached the six-helix bundle stage and C34 still inhibits fusion. Interestingly, 2F5 and 4E10 followed the same inhibition kinetics as C34. The similar values of $t_{1/2}$ for 2F5, 4E10, and C34 indicate that the function of MPER in the fusion cascade is still in effect at a late stage in the fusion reaction (Figure 1).

It has been shown that 2F5 is immunoreactive with free Env-expressing cells and Env-target cell clusters but not with

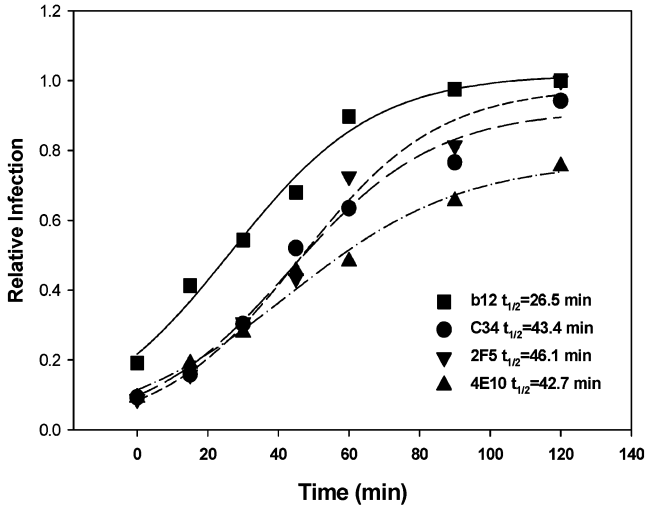

FIGURE 1: Inhibition of HIV-1 infection. Relative inhibition was determined at time 0 and at different times after the incubation of virus with cells at 37 °C in the presence of 50 μg/mL IgG-b12 (■), 50 μg/mL 2F5 (▼), 50 μg/mL 4E10 (▲), or 2 μM C34 (●). Data are representative of an assay done in duplicate and are representative of three independent experiments giving similar results. The half-maximal fusion, $t_{1/2}$, was calculated to be equal to 26.5, 46.1, 42.7, and 43.4 min, respectively, for b12 (solid line), 2F5 (long-dashed line), 4E10 (dashed-dotted line), and C34 (short-dashed line).

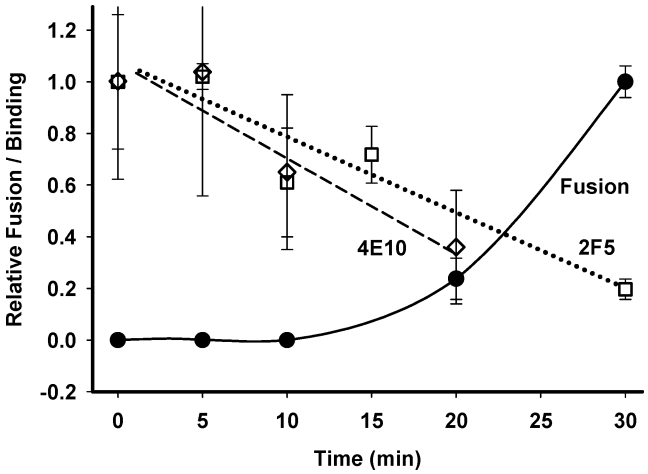

FIGURE 2: Binding of 2F5 and 4E10 to triggered HIV-1 gp41 during the fusion process. HIV-1_{IIIB} Env-expressing CHO cells were incubated with CD4 and CXCR4 bearing SupT1 cells at 37 °C. The binding of 2F5 (□) and 4E10 (◇) and fusion (●) were quantified at different time points as described previously (*20*). The data represent averages of five separate experiments and were normalized relative to mAb binding at time zero or to fusion at 30 min. Smoothed curves passing through the data points of the graphs were generated by a cubic spline interpolation (fusion data) or a linear regression (antibody binding data) using SigmaPlot (SPSS, Inc., Chicago, IL).

fused cells (*23*). In order to further examine the loss of epitope exposure during the fusion reaction, we monitored binding of mAbs, 2F5 and 4E10, following the triggering of Env by appropriate target cells. Figure 2 shows immunofluorescence experiments designed to monitor the binding of 2F5 and 4E10 to HIV-1 upon incubation of HIV-1 Env-expressing CHO cells with SupT1 target cells. The exposure of MPER as measured by mAb binding decreases 2−3-fold within 30 min of incubation of Env-expressing CHO cells with target SupT1 at which time redistribution of calcein

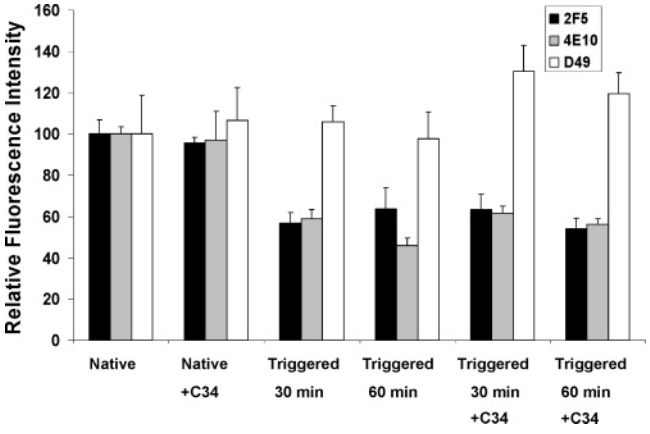

FIGURE 3: Binding of mAbs to triggered HIV-1 gp41 during the fusion process in the presence of C34. HIV-1_{IIIB} Env-expressing cells were incubated at 37 °C with or without SupT1 cells for 30 and 60 min in the presence or absence of 10 nM C34 and examined for 2F5 (black bars), 4E10 (gray bars), and D49 (white bars) mAb binding using the in-cell Western assay with the Odyssey infrared detection system (LI-COR). Nonspecific antibody binding signals were subtracted. Error bars reflect triplicate experiments.

was observed, indicating the onset of fusion. In parallel experiments we have shown that the exposure of gp41 N-HR increases in this time frame (*21*), indicating that the loss of binding to MPER cannot be attributed to a loss of Env from the surface. We attribute the loss of 2F5/4E10 binding to a decrease in the number of Envs with exposed MPER per cell. The remaining fluorescence is then due to binding of the mAbs to Envs that have not yet been triggered. As indicated by the time of addition experiments (Figure 1) total loss of MPER inhibition occurs when the gp41 assumes its fusogenic conformation. In the binding experiments, residual fluorescence is seen in the Envs that have not yet participated in the fusion reaction.

The question therefore arises whether the conformational changes leading to six-helix bundle formation are coupled to MPER function. If this were the case, loss of MPER exposure would not take place in the presence of C34, which inhibits the six-helix bundle formation. We examined this issue further using a different technique, in-cell Western blotting, to monitor 2F5/4E10 mAb binding to HIV-1 gp41. In the case of both mAbs 2F5 (Figure 3) and 4E10 (Figure 3), the epitope exposure is decreased after triggering HIV-1 Env at 30 and 60 min. Figure 3 shows that binding of HIV-1 Env to the D49 mAb, which maps to an epitope in the immunodominant loop of gp41 (*22*), remains unchanged, indicating that the decreases seen with 2F5 and 4E10 are not due to possible removal of gp41 from the surface as a function of time. However, preincubation with the entry inhibitor C34 at 10 nM did not affect the binding, indicating that the two processes are not coupled.

DISCUSSION

The importance of MPER as a target of HIV-1 neutralizing monoclonal antibodies (mAbs) led to an extensive study of the structure and function of this region (*24*). Mutational analyses of MPER show that this region is an important partner together with the six-helix bundle in the overall fusion mechanism (*15*, *16*). Our time of addition studies (Figure 1) indicate that the virus is still susceptible to 2F5/4E10 neutralization at a late triggered state of Env, indicating that these mAbs still bind to MPER at this stage of the game. However, upon triggering we observe a reduction in the number of Envs per cell, whose MPER is accessible to 2F5 and 4E10 (Figure 2). Do these changes reflect different conformations of MPER that occur during infection?

One of the early crystal structures of the HIV gp41 ectodomain in isolation includes the initial portion of the 2F5 epitope (*5*). This structure presumably represents the ectodomain in a postfusion conformation and shows that the initial sequence of the MPER is helical under these conditions. The NMR structure of the SIV gp41 ectodomain also confirms the helical structure of the initial residues of the MPER in the six-helix bundle (*2*). Peptides from gp41 of moderate length bind with high affinity to 2F5 [e.g., ELLELDKWASLWN (*25*)]. The core of the 4E10 epitope, NWFDIT, maps just C-terminal to the 2F5 epitope on the gp41 ectodomain (*26*, *27*). Although the structures of gp41 MPER-derived peptides have extensively been studied in aqueous solution (*25*, *28*), in lipid micelles (*29*) and in complex with mAbs (*30*, *31*), the structure of the MPER in the context of the entire gp120-gp41 is not known. However, the NMR structures of the peptides taken together indicate that MPER can assume a helical conformation independent of six-helix bundle formation, consistent with our findings that changes in the exposure of the MPER are not dependent upon six-helix bundle formation (Figure 3).

On the basis of the extended conformation of the 2F5 epitope, whose crystal structure has been determined in association with the 2F5 mAb (*31*), one could argue that this region undergoes a conformational transition to a helical form after triggering the fusion reaction. On the other hand, the 4E10 epitope, whose crystal structure has been determined in association with the 4E10 mAb (*30*), is helical from the outset. Biochemical studies with proteoliposomes (*31*, *32*) indicate the importance of lipid membrane and hydrophobic context in the binding of 2F5 and 4E10. Moreover, biophysical studies with MPER peptides indicate that the role played by MPER in the fusion reaction may be due to the strong interaction of this region with the membrane of the virus (*33*, *34*).

Since optimal binding of 2F5/4E10 occurs in the presence of lipid (*31*, *32*), we believe that MPER−lipid membrane interactions per se are not responsible for the loss of reactivity during the fusion reaction (Figures 2 and 3). We hypothesize that the loss of accessibility of 2F5/4E10 to MPER may be due to an inward curvature of the viral membrane which wraps around the MPER epitope (see table of contents graphic). However, the loss of the MPER reactivity is not counteracted by the presence of the six-helix bundle inhibitor, C34 (Figure 3), indicating that this effect is not coupled to six-helix bundle formation. The presumed inward curvature may therefore be caused by the mechanics of the virus−cell or cell−cell interactions which occur upon binding of CD4 to gp120 (see table of contents graphic).

In conclusion, our studies with the mAbs against the MPER region have provided mechanistic insight into HIV-1 fusion. The two important events in the fusion reaction, six-helix bundle formation and MPER function, occur in a similar time frame, but they do not appear to be coupled. These data need to be taken into consideration in the design of models that explain HIV-1 Env-mediated fusion.

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