HIV gp120-induced Interaction between CD4 and CCR5 Requires Cholesterol-rich Microenvironments Revealed by Live Cell Fluorescence Resonance Energy Transfer Imaging*

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Binding of the human immunodeficiency virus (HIV) envelope gp120 glycoprotein to CD4 and CCR5 receptors on the plasma membrane initiates the viral entry process. Although plasma membrane cholesterol plays an important role in HIV entry, its modulating effect on the viral entry process is unclear. Using fluorescence resonance energy transfer imaging, we have provided evidence here that CD4 and CCR5 localize in different microenvironments on the surface of resting cells. Binding of the third variable region V3-containing gp120 core to CD4 and CCR5 induced association between these receptors, which could be directly monitored by fluorescence resonance energy transfer on the plasma membrane of live cells. Depletion of cholesterol from the plasma membrane abolished the gp120 core-induced associations between CD4 and CCR5, and reloading cholesterol restored the associations in live cells. Our studies suggest that, during the first step of the HIV entry process, gp120 binding alters the microenvironments of unbound CD4 and CCR5, with plasma membrane cholesterol required for the formation of the HIV entry complex.

Entry of human immunodeficiency virus-1 (HIV-1)2 into cells requires the formation of entry complexes involving the viral envelope glycoprotein gp120 and the target cell receptors CD4 and either CCR5 or CXCR4. CCR5 and CXCR4 are G-protein-coupled chemokine receptors that play critical roles in immune responses (1–4). Considerable effort has been focused on the development of therapeutics targeting chemokine receptors to block HIV entry (5, 6). A major problem in developing safe and effective co-receptor inhibitors is the risk of harmful side effects. Understanding the molecular mechanisms underlying chemokine function in HIV infection will hopefully provide a foundation for designing and screening drugs that offer strong and long lasting HIV-inhibitory function but have little effect on the physiology of the homeostatic chemokine system. In the past years, extraordinary progress has been made in solving the structures of gp120 and CD4 and in demonstrating that chemokines and small derivative molecules block HIV infection (4, 6–11). Yet, the details regarding gp120-induced formation of HIV entry complexes in the context of live cells still need to be resolved.

The plasma membrane of eukaryotic cells consists of a complex assembly of various lipids and proteins that are distributed in regions of distinct lipid microenvironmments known as lipid or non-lipid raft microdomains (12–17). Lipids in rafts possess long and saturated fatty acyl chains and are organized in a tightly packed, liquid-ordered manner, whereas non-lipid raft microdomains contain shorter, unsaturated fatty acyl chains and are in a loosely packed, disordered state (12–17). Lipid rafts are defined as microdomains that are enriched with cholesterol, glycosphingolipids, and sphingomyelin and are often isolated in detergent-resistant membrane fractions. Previous studies draw different conclusions regarding the role of lipid raft microdomains during the formations of HIV entry complexes involving CD4 and CCR5. Using detergent insolubility or immunostaining with fluorescence microscopy, several studies suggest that CD4 and CCR5 receptors localize in lipid rafts, and this localization is important for ligand binding, receptor signaling, and the formation of HIV entry complexes (18–20). In contrast to these reports, other reports suggest that the localization of CD4 and CCR5 in the lipid raft microdomains is not required for HIV infection and conclude that HIV entry into T-cells does not depend on the localization of CD4 and CCR5 to cholesterol-enriched, detergent-resistant membrane microdomains (20, 21). However, recent studies suggest that membrane microdomains isolated from detergent do not reliably reflect the organization of the lipids in the cell membrane. It has been very difficult to demonstrate the existence of lipid rafts in cells, because their size is <70 nm in diameter, which is too small to be resolved by light microscopy. In addition, their stability and motion on the plasma membrane of live cells are unclear (12, 14, 15, 22). Because of the dynamic and submicroscopic nature of lipid microdomains in living cells, the physiological function of lipid microdomains in HIV entry is still in dispute.

structural studies suggest that a mature HIV particle contains an average of 10–15 spikes, the distance between two spikes is ~23 nm, and a spike forms a knob with a diameter of 10.5 nm (23–26). Binding of CD4 to gp120 leads to conformational changes in a spike that allows gp120 to interact with a
chemokine receptor. Binding of gp120 to CD4 and a chemokine receptor triggers further structural changes that allow the gp41 to insert into the target cell membrane (1, 4). In the current model, one gp120 interacts with one CD4 and one chemokine receptor. Evidence from immunoelectron microscopy (27) demonstrates that, before contacting HIV, CD4, CXCR4, and CCR5 each form independent microclusters that are separated by a distance of ~10 nm. Based on x-ray crystal structures and immunoelectron microscopy studies (9, 10, 27, 28), the distance between CD4 and the chemokine receptors is estimated to be well within 10 nm when a HIV entry complex is formed. High resolution fluorescence imaging techniques that take advantage of fluorescence resonance energy transfer (FRET) between fluorescence proteins have been developed that offer a new approach for studying the formation of HIV entry complex in living cells (29). Because of the extreme sensitivity of the efficiency of FRET to the distance between FRET donor and acceptor proteins, which are within 10 nm, FRET imaging has proven to be a valuable tool for studying protein-protein interaction as well as conformational changes in a protein complex in live cells (30–33).

Here we report the results of studies using FRET imaging coupled with quantitative microscopy to monitor gp120-induced interactions on the plasma membrane of live cells between CCR5 and CD4, which were tagged with cyan fluorescent protein (CFP) (FRET donor) and yellow fluorescent protein (YFP) (FRET acceptor). We observed that gp120 variants that interact with both CD4 and CCR5 induced FRET increases. Disruption of lipid raft microenvironments by methyl-β-cyclodextrin (MβCD), which depletes cholesterol, did not alter the distributions of CD4 and CCR5 on the membrane. However, this treatment blocked gp120-induced FRET increases, suggesting that cholesterol-rich microdomains are required for gp120-induced interactions between CD4 and CCR5.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—pEYFP-N1 and pECFP-N1 were purchased from Clontech (Palo Alto, CA). Lipofectamine 2000 was purchased from Invitrogen. HIV-1 core + V3 (JRFL, YU2, HXB2) (36), SDF1α, and RANTES were purchased from BIO-SOURCE (Camarillo, CA). Fluo-4-AM was from Molecular Probes (Eugene, OR). Water-soluble cholesterol was from Sigma-Aldrich. Anti-GFP monoclonal antibody (JL-8) was from BD Biosciences. All of the other reagents were reagent grade and were obtained from standard suppliers.

**Plasmid, Cell Line, and Transfection**—Human CD4 and CCR5 genes were generated by PCR. The plasmids encoding CD4-YFP were constructed by inserting the PCR product of CD4 into the pEYFP-N1 vectors between the BglII and EcoRI sites. The plasmid encoding CCR5-CFP was constructed by inserting the PCR product of CCR5 into the pECFP-N1 vector between the HindIII and BamHI sites. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum (10%), penicillin (5 μg/ml), and streptomycin (5 μg/ml) and were grown in 5% CO2 at 37 °C. The HEK293T cells were transfected or co-transfected with the plasmids encoding CD4-YFP and/or CCR5-CFP mediated by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Cholesterol Extraction and Reconstitution**—For cholesterol extraction, cells in serum-free medium were treated with 10 mM MβCD for 30 min at 37 °C and then rinsed and incubated in the growth medium with 1% lipid-free bovine serum albumin. The efficiency of cholesterol extraction was reported in previous studies (30). MβCD-treated cells were reconstituted with 100 μg/ml water-soluble cholesterol at 37 °C for 1 h.

**Calcium Assay**—HEK293T cells were seeded in four-well chambers at 10E4/ml, 24–36 h before the experiments. After 3 h of starvation, the cells were labeled by incubation with Fluo-4-AM in Hanks’ balanced salt solution for half an hour, washed twice, and incubated for half an hour before imaging under the microscope. Upon the addition of gp120 or RANTES (regulated upon activation normal T cell-expressed and -secreted) (50 nm) to the cell chamber, time-lapse images were collected in multitrack mode, and CFP and Fluo-4 images were digitally separated. Changes in Ca2+ concentration were monitored as the changes in the intensity of Fluo-4 (I/Ip, where I is the intensity at time t and Ip is the intensity at time 0).

**Imaging and FRET Assay**—Cells were washed twice with 1× Hanks’ balanced salt solution and then starved in 1× Hanks’ balanced salt solution plus 1% bovine serum albumin for 3 h. Before the imaging, the cells were treated with 0.15 nm gp120 (JRFL, YU2, HXB2) for 20 min. A Zeiss Plan-apochromat 40× oil immersion objective was used for image acquisition. Images were collected in multitrack mode (Zeiss 510). In Track I, there were two channels, cells were excited with 458 nm, CFP emission signals were collected through Channel I (475–525 nm), and FRET emission signals were collected through Channel II (>530 nm). In Track II, there was only one YFP channel, and YFP emission signals were collected with this channel (>530 nm). FRET efficiency between CFP and YFP was analyzed using Zeiss laser scanning microscope software.

Intermolecular FRET efficiency was shown as normalized FRET (NFRET) using macros of Zeiss LSM software. Briefly, correction factors were determined from single CFP or YFP control cells as follows: β(F/D) from CCR5-CFP single positive cells and γ(F/A) and δ(D/F) from CD4-YFP single positive cells, where F, D, and A are fluorescence intensities of the donor (CFP) when excited with the wavelength of the donor (CFP) channel, that of the acceptor when excited with wavelength of the donor (FRET) channel, and that of the acceptor when excited with the wavelength of the acceptor (YFP) channel. β, γ, and δ depend on the image acquisition settings and were determined under identical settings for all FRET experiments. The original images were background-subtracted, and CFP images were further corrected for bleed-through components consisting of fractions of actual acceptor fluorescence at 458 nm excitation by image processing using the equation Dcor = D − δF/1 − βδ. NFRET images were processed on a pixel-by-pixel basis using equation NFRET = [F − βD − γ(1 − βδ)A]/1 − βδ/((Dcor)A)1/2 (38). For quantification of NFRET, regions of interest (ROIs) covering the plasma membrane from the acquired images were chosen, processed as above, and calculated automatically in the FRET macro of the LSM imaging software pro-
molecular mass by Western blotting using anti-GFP antibodies (Fig. 1, C and F). Two bands were detected by anti-GFP antibodies in the cells expressing CCR5-CFP. The upper one is CCR5-CFP and the lower one, ~30 kDa, is likely the CFP portion of the degraded products of CCR5-CFP, which is typical of ectopically expressed chemokine G-protein-coupled receptors (30).

**Ligand of CCR5 Triggers a Ca\(^{2+}\) Response Mediated by CCR5-CFP—** To test the functionality of the CCR5-CFP fusion protein, we examined the ligand-induced Ca\(^{2+}\) response in the CCR5-CFP-expressing cells (Fig. 2). We imaged a fluorescence intensity change of Fluo-4 (a fluorescent calcium indicator) triggered by MIP1\(\alpha\) (a ligand for CCR5). Using a confocal fluorescence microscope (Zeiss 510 META), fluorescence images of Fluo-4 (Fig. 2A, green) and CCR5-CFP (red) were simultaneously recorded in a time-lapse experiment. Fluo-4 was distributed throughout the entire cytosol, and CCR5-CFP was uniformly distributed on the cell surface. Upon the addition of MIP1\(\alpha\) to the cell chamber, the green fluorescence signal transiently increased in the cytosol, indicating that MIP1\(\alpha\) induced changes in Ca\(^{2+}\) concentration (Fig. 2). The distribution of CFP signal (Fig. 2, red) was not significantly changed upon stimulation, suggesting that ligand binding immediately triggered downstream signaling events without altering the membrane distribution of CCR5. To ensure that the MIP1\(\alpha\)-elicited Ca\(^{2+}\) responses were specifically mediated by the expressed CCR5-CFP, we did a control experiment with the parental HEK293 cells. No apparent increase in green fluorescence signal was observed after MIP1\(\alpha\) stimulation (Fig. 2B). Other ligands of CCR5, such as RANTES, also triggered CCR5-CFP-mediated Ca\(^{2+}\) response (data not shown). These results demonstrated that the CCR5 receptor fused with CFP at its C terminus retained its functions in ligand binding and in signaling. CD4 fused with CFP or YFP at the C terminus has been previously shown to be functional in T-cell receptor signaling (34, 35). Therefore, cells expressing CCR5-CFP and CD4-YFP provide a system for probing extracellular ligand-induced changes in the dynamic distribution of these receptors on the plasma membranes of live cells.

**RESULTS**

**Expression of CCR5 and CD4 Receptors Tagged with Fluorescence Proteins—** To investigate the arrangement of CCR5 and CD4 in the plasma membranes of live cells, we fused CFP to the C terminus of CCR5 and YFP to the C terminus of CD4. HEK293 cells were transfected with the construct expressing CCR5-CFP or CD4-YFP (Fig. 1). CCR5-CFP (Fig. 1B) and CD4-YFP (Fig. 1C) were expressed efficiently on plasma membrane with no gross heterogeneity in their distribution. CCR5-CFP and CD4-YFP were detected with the expected

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**FIGURE 1. Expression of CFP- or YFP-tagged CCR5 or CD4 receptors.** A, schematic diagram of CCR5-CFP on the cell membrane. B, confocal images of living cells expressing membrane-localized CCR5-CFP (cyan). Top panel, fluorescence image. Bottom panel, the merged image of the fluorescence and differential-interference-contrast channels. Scale bar, 10 \(\mu\)m. C, fusion protein of CCR5-CFP was detected by Western blotting in whole cell lysate using an anti-GFP antibody. Lane 1, HEK293 cells (control); lane 2, HEK293 cells transiently expressing CCR5-CFP. D, schematic diagram of CD4-YFP on the cell membrane. E, confocal images of living cells expressing membrane-localized CD4-YFP (yellow). Top panel, fluorescence image; middle panel, differential-interference-contrast image; bottom panel, merged image. Scale bar, 10 \(\mu\)m. F, fusion protein of CD4-YFP was detected by Western blotting using an anti-GFP antibody. Lane 1, HEK293 cells (control); lane 2, HEK293 cells transiently expressing CD4-YFP.

**FIGURE 2. Ligand of CCR5-induced Ca\(^{2+}\) response in living cells expressing CCR5-CFP.** A, CCR5-CFP (red) mediated a transient intracellular Ca\(^{2+}\) elevation and is visualized as intensity changes of Fluo-4 (green). MIP1\(\alpha\) was added at time 0. \(\beta\), MIP1\(\alpha\)-induced Ca\(^{2+}\) response in living cells. A time course of intracellular Ca\(^{2+}\) changes following the addition of MIP1\(\alpha\) is shown. The 20-s time point is shown as the intensity changes of Fluo-4 in HEK293 cells (as a control (gray)) and in the cells expressing CCR5-CFP (black). Means ± S.D. are shown (n = 8).
Aggregation of CD4-YFP on Plasma Membrane Induced by gp120 and anti-CD4 Antibodies—We investigated membrane distribution of CD4-YFP in the cells stimulated with anti-CD4 antibody and two different variants of gp120. The V3-containing gp120 core of HXB2 variant binds CD4 and CXCR4 but not CCR5, and the V3-containing gp120 core of JRFL variant binds CD4 and CCR5 but not CXCR4 (36). CD4-YFP was uniformly distributed on the plasma membrane before stimulation (Fig. 3B, Control). After incubation with anti-CD4 antibody (CD4-YFP, 20 nM) for 30 min, the third variable region V3-containing gp120 core of JRFL (20 nM) and the V3-containing core of HXB2 (20 nM) formed large patches on the plasma membrane, indicating CD4 binding to anti-CD4. JRFL and HXB2 also induced aggregation of CD4-YFP on the plasma membrane. We then studied whether the CCR5 receptor affects these ligand-induced CD4 aggregations. Cells expressing both CD4-YFP and CCR5-CFP were used for the live cell experiment (Fig. 3, C and D). Before stimulation, both CD4-YFP and CCR5-CFP were uniformly distributed on the plasma membrane. After incubation for 30 min with HXB2 and anti-CD4, both of which bind to CD4 but not CCR5, CD4-YFP clearly aggregated on the plasma membrane, whereas CCR5-CFP remained uniformly distributed. Interestingly, incubation of JRFL, which binds both CD4 and CCR5, did not induce a clear CD4-YFP aggregation. One possible explanation for the gp120-induced aggregation is that a small amount of gp120 forms oligomers leading to CD4 aggregation. Another explanation is that gp120 binding may trigger intracellular signaling that results in the aggregation. Although we do not have a clear explanation for the phenomenon, our observations combined with previous studies (27, 37) suggest that CD4 and CCR5 do not form stable heterodimers in the absence of extracellular ligands that bind both of them.

Measurement of FRET between CD4-YFP and CCR5-CFP—To assess interactions between CD4 and CCR5, we measured FRET between CFP and YFP in cells co-expressing CD4-YFP and CCR5-CFP. We used the sensitized emission method to calculate FRET between CD4-YFP and CCR5-CFP using confocal microscopy. Using a multitrack and line-scanning mode of a laser scanning confocal microscope, cells were simultaneously recorded in the CFP, YFP, and FRET detection channels. Fluorescence was simultaneously collected pixel-by-pixel from three detection channels: CFP (458 nm,
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A schematic diagram of FRET measurement between CFP-tagged CCR5 and YFP-tagged CD4. When cells were excited at 458 nm, emissions were simultaneously recorded in the CFP and FRET channels; when cells were excited at 514 nm, emissions were recorded in the YFP channel. We used multichannel and line scanning mode so that images of three channels were recorded simultaneously. Cells expressing only CCR5-CFP or CD4-YFP were used as controls for calculating real FRET efficiency (NFRET). B, images of cells expressing CCR5-CFP, CD4-YFP, or both CCR5-CFP and CD4-YFP without or with gp120 (a YU2 variant that binds both CCR5 and CD4). NFRET in the plasma membrane was measured in different ROIs, which are indicated in the FRET channel. NFRET images show that FRET intensity increased when the cell was stimulated with YU2 (gp120). C, NFRET value is relatively independent of the concentration of CFP or YFP intensity in the ROI. These values are from many membrane regions of multiple cells.

Figure 4. An analysis of CCR5 and CD4 interaction by FRET imaging. A, schematic diagram of FRET measurement between CFP-tagged CCR5 and YFP-tagged CD4. When cells were excited at 458 nm, emissions were simultaneously recorded in the CFP and FRET channels; when cells were excited at 514 nm, emissions were recorded in the YFP channel. We used multichannel and line scanning mode so that images of three channels were recorded simultaneously. Cells expressing only CCR5-CFP or CD4-YFP were used as controls for calculating real FRET efficiency (NFRET). B, images of cells expressing CCR5-CFP, CD4-YFP, or both CCR5-CFP and CD4-YFP without or with gp120 (a YU2 variant that binds both CCR5 and CD4). NFRET in the plasma membrane was measured in different ROIs, which are indicated in the FRET channel. NFRET images show that FRET intensity increased when the cell was stimulated with YU2 (gp120). C, NFRET value is relatively independent of the concentration of CFP or YFP intensity in the ROI. These values are from many membrane regions of multiple cells.

CFP excitation; 475–525 nm, CFP emission; FRET (458 nm, CFP excitation; long pass filter 530 nm, YFP emission); and YFP (514 nm, YFP excitation; long pass filter 530 nm, YFP emission) (Fig. 4A). We first obtained bleed-through (cross-talk) co-efficients by analyzing images from cells expressing only CCR5-CFP or CD4-YFP, which were imaged with the identical configuration and scanning setup as the controls (Fig. 4B). We then obtained FRET efficiency and FRET images from cells expressing both CCR5-CFP and CD4-YFP. Using the FRET analysis tool, the FRET macro for the Zeiss LSM 510 META microscope, the NFRET image with intensities was converted from the FRET index calculated for each pixel using the Xia method (38) (details under “Experimental Procedures”).

A major advantage of using confocal microscopy to evaluate FRET is that individual ROIs within a cell can be selectively examined for FRET efficiency. In contrast, flow cytometric or fluorometric approaches can only measure total cellular FRET, which often includes high concentrations of fluorophores in intracellular compartments. Because we were interested in CCR5 and CD4 interactions at the cell surface, only the plasma membrane region of the cell was used as the ROI. Although CCR5-CFP and CD4-YFP both localized on the cell membrane, very low FRET intensity was detected in the cells before the addition of gp120 (Fig. 4B). Strikingly, after incubation of gp120, which binds both CD4 and CCR5, a strong FRET signal was detected on the membrane of these cells. FRET efficiency is usually sensitive to the relative amounts of donors and acceptors in the selected regions. However, our method calculated a corrected FRET value by dividing the square root of the donor and acceptor concentrations pixel-by-pixel and therefore providing a reliable FRET measurement, as it depended less on the concentra-
determine the relationship between gp120-induced FRET changes and specificity in interactions between gp120 and CD4 or CCR5, we measured the FRET efficiency in CD4-YFP/CCR5-CFP-expressing cells in the presence of V3-containing gp120 cores of JRFL and YU2 (variants that bind to CCR5) and the core of HXB2 (a variant binds to CXCR4) (Fig. 5). In control experiments, NFRET values in cells that were not incubated with any ligand or stimulated with SDF1α, a ligand for CXCR4, were relatively low. A small and insignificant increase in FRET was detected in cells that were incubated with HXB2. In contrast, large (~40%) FRET increases were detected in cells that were stimulated with JRFL and YU2 (Fig. 5). Quantitative analyses of FRET efficiency indicated that there is a statistically significant increase in FRET in cells that were treated with JRFL and YU2. Our results indicated that gp120, which interacts with both CD4 and CCR5, could bring CD4 and CCR5 together on the plasma membrane of live cells.

**Effect of Cholesterol Depletion and Reloading on gp120-induced FRET Increase**—To probe the requirements for gp120-induced interaction between CD4 and CCR5, we treated cells with MβCD to remove cholesterol from the membrane and to disrupt the function of the cholesterol-rich membrane microdomain (Fig. 6) (12–17, 30). When cells were treated with MβCD, we detected a low level of FRET, which was similar to that in cells that were not stimulated. After MβCD treatment, JRFL and YU2 did not induce any FRET increase, although the levels of CD4-YFP and CCR5-CFP and their distributions remained same. If cholesterol was added back to the chamber after the MβCD treatment, either V3-containing cores of JRFL or YU2 could induce FRET increases in the cells, indicating that replenishing cholesterol to the plasma membrane restores the membrane requirements for gp120-induced interaction between CD4 and CCR5. Moreover, if cells were incubated with V3-containing cores of JRFL and YU2 first and then treated with MβCD to remove cholesterol from the membrane, FRET value remained low. These results suggest that gp120-induced CD4-CCR5 interaction is a dynamic process and cholesterol-rich microdomains are required for maintaining the interactions on the plasma membrane.

**DISCUSSION**

In this study, we used a live cell system to investigate gp120-induced interaction between CD4 and CCR5, and our results have provided new insights on this issue in comparison with previous studies of this earliest step of the formation of HIV entry complex by conventional imaging and biochemical approaches. Previous studies have demonstrated that depletion of plasma membrane cholesterol inhibits HIV entry. However, the modulating effect of plasma membrane cholesterol on the HIV entry process has been controversial. In one view, the removal of cholesterol disrupts lipid rafts where HIV forms the entry complex, thereby inhibiting HIV entry (18–20). In another view, the formation of the HIV entry complex takes place outside of lipid rafts, and cholesterol modulates the HIV entry process independently of its ability to promote lipid raft formation (20, 21). There are several lines of evidence arguing for the second view. CCR5 receptors are not largely present in the detergent-resistant membrane fraction, which is the bio-
chemical definition of lipid rafts. Although CD4 receptors are enriched in detergent-resistant membrane, a mutant CD4 that does not stay in detergent-resistant membrane is still able to mediate HIV infection. It has become increasingly clear that plasma membrane contains various microenvironments. The operational definitions for lipid rafts, such as detergent insolubility to define components of rafts, cholesterol depletion to define raft functions, and immunostaining to define co-localization with rafts on the cell surface are not adequate to describe the dynamic nature and the complexity of microenvironments in native cell membrane. On the other hand, live cell FRET approaches provide a powerful tool to probe the dynamic distribution of proteins in plasma membrane.

Our current results indicate that CCR5 and CD4 localize in different and small microenvironments, although both of them are uniformly distributed on the plasma membrane. We found that anti-CD4 antibody or gp120 that binds to CD4 (but not to CCR5) induced an aggregation of CD4 (but not of CCR5) on the plasma membrane. We also found that CD4-YFP and CCR5-CFP each displayed a different lateral diffusion on the plasma membrane using FRAP (fluorescence recovery after photobleaching) analysis (data not shown). Different dynamic properties suggested that these two receptors are spatially separated in the resting state, which is consistent with previous studies by FRAP, immunoelectron microscopy, and biochemical approaches (27, 37). Although some studies suggest that both CD4 and CCR5 are localized within raft regions and a stable association exists between CD4 and CCR5 in the plasma membrane (40), our results and previous studies (27, 37) indicate that CD4 and CCR5 move independently with one another in the membrane before they encounter extracellular ligands.

The gp120 variants JRFL and YU2 that bind extracellular domains of both CD4 and CCR5 induced a clear FRET increase between CCR5-CFP and CD4-YFP, suggesting that our FRET measurement was sensitive enough to monitor the formation of the protein complex of gp120, CD4, and CCR5 on the plasma membrane of live cells. FRET can be used to calculate the distance between the donor and acceptor and thereby infers the distance between the molecules to which they are linked. At this stage, it is difficult to calculate the precise distance between CD4 and CCR5, because the relative orientations of CFP and YFP are unknown in CD4-YFP and CCR5-CFP. Future structural studies may provide information of their orientations in these tagged receptors, which will allow us to calculate the distance without assuming a free rotation of CFP and YFP, although it is unlikely to be the case when they are tethered to CD4 and CCR5. However, FRET between CFP and YFP is detectable only within the distance of 10 nm, and FRET efficiency increases exponentially as the distance between fluorophores decreases. Thus, our data indicate that CFP and YFP are held in extremely close proximity upon gp120 binding to CD4 and CCR5.

The FRET increase induced by gp120 requires cholesterol in the plasma membrane, suggesting that the protein complex of gp120, CD4, and CCR5 forms in a cholesterol-rich microenvironment in live cells. We propose that gp120 binding to CD4 and CCR5 triggers the conformational changes that alter their affinity to different lipids in the plasma membrane, leading to a partitioning of the complex with cholesterol-rich microenvironments. This cholesterol-rich microenvironment differs from the lipid raft microdomains (where unbound CD4 localizes) and also differs from non-lipid raft microdomains (where...
unbound CCR5 resides). The idea that unbound CD4 and CCR5 and gp120-bound CD4 and CCR5 change their microenvironments is consistent with previous studies by biochemical approaches and also reflects the dynamic and complicated nature of the plasma membrane.

The live cell system with FRET imaging analysis developed in the present study allows direct monitoring of the formation of gp120, CD4, and CCR5, the first step of the HIV entry process. Many questions regarding the development of safe and effective inhibitors targeting this step of HIV infection can now be addressed directly. Future studies on developing and applying this new technology to the study of HIV opens the possibility of the elucidation of the molecular details of the HIV entry mechanisms. Such knowledge could lead to new strategies for designing and screening drugs against HIV infection.

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