CD4 Interacts Constitutively with Multiple CCR5 at the Plasma Membrane of Living Cells

A FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING AT VARIABLE RADII APPROACH

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The entry of human immunodeficiency virus into target cells requires successive interactions of the viral envelope glycoprotein gp120 with CD4 and the chemokine receptors CCR5 or CXCR4. We previously demonstrated, by Förster resonance energy transfer experiments, the constitutive association of CD4 and CCR5 at the surface of living cells. We therefore speculated that this interaction may correlate with compartmentalization of CD4 and CCR5 within the plasma membrane. Here, we characterize the lateral distribution, the dynamics, and the stoichiometry of these receptors in living cells stably expressing CD4 and/or CCR5 by means of fluorescence recovery after photobleaching at variable radii experiments. We found that (i) these receptors expressed alone are confined into 1-μm-sized domains, (ii) CD4-CCR5 associations occur outside and inside smaller domains, and (iii) these interactions involve multiple CCR5 molecules per CD4.

The fusion induced by human immunodeficiency virus type 1 (HIV-1)6 is a multistep process that requires for the virus to bind to two different host cell surface receptors. Current models suggest an initial interaction between the trimeric viral envelope protein gp120 and the primary CD4 receptor on target cells. This results in a conformational change of gp120 that exposes a co-receptor binding site. After binding to the co-receptor molecule, either CXCR4 or CCR5 depending on virus tropism, additional structural changes take place, allowing the viral gp41 protein to initiate the fusion of cellular membranes. This last step leads to the release of the viral genome into the cytoplasm of the target cell (for a review, see Ref. 1).

A more efficient co-immunoprecipitation of CD4 with CCR5 compared with CXCR4 supports the existence of preferential interactions between CCR5 and CD4 (2), which could be at the origin of the predominance of R5-tropic HIV. In addition, a multiplicity of interactions are involved, since the attachment of several gp120 envelope trimers to multiple CD4 (3) and to multiple CCR5 co-receptors (4) seems to be needed for infection to proceed (5).

Thus, the fusion process involves the encounter and interaction of multiple receptors at the plasma membrane. A compartmentalization of the partners could favor this step of the infection. The virus may take advantage of a clustering to rapidly form multivalent interactions with the receptors and co-receptors required for fusion. The heterogeneous patchiness of the membrane can account for such an organization. Indeed, the current view of the plasma membrane is a “dynamically structured mosaic model” (6) characterized by a dynamic organization into domains scaling from the nanometer to micrometer range (7, 8). Evidence is accumulating that compartmentalization of membrane components could be essential for the regulation of cellular functions (7).

In the case of CD4 and CCR5, several results suggest the existence of such a compartmentalization. First, electron microscopy observations show that CCR5, CXCR4, and CD4 form homogeneous microclusters at the surface of primary macrophages and T cells (9). Second, our recent study carried out using a steady state Förster resonance energy transfer (FRET) approach, at the surface of living cells, demonstrated the constitutive interaction of CD4 and CCR5, reinforced by gp120. This finding indicated that a ternary complex between gp120, CD4, and CCR5 would form before the fusion process occurred (10).

The kinetics of the successive interactions leading to the virus attachment is an important point of this process; the interaction of gp120 with CCR5 must take place within the lifetime of the gp120-CD4 bound. Due to the short lifetime of this interaction (11), CD4 and CCR5 must encounter very fast. This condition can be fulfilled by either a rapid diffusion or a close localization of the receptors at the membrane.

Currently, data on dynamic and membrane organization of receptors are still incomplete, and a thorough analysis of the
CD4 Interacts Constitutively with Multiple CCR5

dynamic behavior of the HIV co-receptors within the membrane is needed for a better understanding of the early steps of the infection process.

To investigate the diffusion behavior of CD4 and CCR5 in the membrane of living HEK293T cells, we exploited vrFRAP, a powerful approach used to identify and characterize membrane domains (12, 13). We reveal a microcompartimentalization of CD4 and CCR5. We show further evidence for a specific interaction between CD4 and CCR5 receptors at the basal state and demonstrate that these interactions involved several CCR5 per CD4. Finally, for the first time, we highlight the dynamics of this prefusion complex within the membrane of living cells.

EXPERIMENTAL PROCEDURES

Chemicals—sCD4 and gp120 were obtained from the AIDS Research and Reference Reagent Program catalog of the National Institutes of Health (Bethesda, MD). The fluorescent lipid didodecylphosphatidylethanolamine-7-nitrobenz-2-oxa-1,3-diazole-4-yl (dC12-PE-NBD) was synthesized as previously described (14).

Tagged and Wild-type CD4 and CCR5 cDNA Constructs—cDNAs encoding wild-type CD4 and CCR5 receptors were inserted into eukaryotic expression vectors pcDNA 3.1 hygro between the HindIII and XbaI restriction sites. The cDNAs encoding the tagged receptors with the T7 tag at the N terminus and eGFP at the C terminus (10, 13) were inserted into pRC/CMV expression vectors (Invitrogen) between the HindIII and XbaI restriction sites. The following plasmids were obtained: pcDNA 3.1 hygro/wtCCR5, pcDNA 3.1 hygro/wtCD4, pRC/CMV-SP/a7AChR-T7-CCR5-eGFP, and pRC/CMV-SP/a7AChR-T7-CD4-eGFP.

Cell Culture and Transfections—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Sigma) at 37 °C and 5% CO₂ atmosphere. The tagged receptors were expressed in these cells by transfection of plasmid (concentrations ranging from 0.25 to 2 μg) using the calcium phosphate-DNA co-precipitation method. For the vrFRAP experiments, cells were plated onto 22 × 22-mm coverslips precoated with polylysine (Poly-L-Lysine; Sigma) and incubated for 5 min at room temperature in a 100 μg/ml aqueous solution. The lipid solution was then dissolved in 100 μl of ethanol, and the mix was injected with a syringe (Hamilton, Switzerland) in 10 ml of phosphate-buffered saline Ca²⁺-Mg²⁺. The final lipid concentration was 40 μM. Cells were incubated with the lipid for 15 min at 20 °C and then washed twice with phosphate-buffered saline Ca²⁺-Mg²⁺ before carrying out the vrFRAP experiments.

Experimental vrFRAP Measurements—Fluorescence recovery curves were measured at 20 °C in order to minimize eventual internalization of tagged receptors using a homemade device (12). The bleaching times were set between 20 and 40 ms, and the recovery was recorded over 20 s with a sampling rate of 2 ms. The radius of the observation area (R) was varied between 1.40 and 3.45 μm. For each experimental condition tested, more than 30 fluorescence recoveries were accumulated on different cells. These series of experiments were carried out at least in triplicate, and all curves set were averaged. The diffusion coefficient (D) and the mobile fraction (M) were estimated with a confidence interval of 95% by fitting the data with the suitable diffusion equation (16), assuming either one or two diffusing populations of tracers.

Conceptual Background of the vrFRAP Data Analysis—vrFRAP is a powerful approach for studying membrane dynamic organization. Monte Carlo simulations and experiments on a model of compartmentalized membranes showed that the analyses of the variation of M and D with R allow one to characterize membrane compartmentalization (17) as follows. (i) In the absence of domains, M and D are independent of R, and the measured diffusion coefficient corresponds to the real one. (ii) If the membrane is compartmentalized, then M and D are dependent on R. For a domain size L < R, the following relationship is expected,

\[ M = M_p + 0.63L/R \]  

(Eq 1)

where \( M_p \) is the permanent mobile fraction. For domain sizes \( L > R \), the mobile fraction reaches a limit value called \( M_p \), and \( M_f \) can be roughly estimated by the value of the mobile fraction at \( R \sim L \) (see Fig. 1).

Depending on the \( M_p \) value, two cases can be considered. (i) If \( M_p \approx 0 \), there is a single population of tracers confined in
closed domains. The measured diffusion coefficient is an apparent one \( D_{\text{app}} \), and the real diffusion coefficient inside the domains \( D_{\text{conf}} \) can be evaluated from Equation 2.

\[
D_{\text{conf}} = \frac{1}{2} D_{\text{app}} \left( \frac{L}{R} \right)^2
\]

(Eq. 2)

The analysis must be validated by verifying that \( D_{\text{conf}} \) is invariant with \( R \). (ii) If \( M_{\phi} > 0 \), the tracers are either inside joined but open domains or distributed inside and outside of isolated domains. An analysis of the recovery curves assuming two populations of tracers is necessary, which leads to the determination of the weight and diffusion coefficient of each population. One has a measured diffusion coefficient varying according to Equation 2 and corresponds to confined tracers with \( D_{\text{conf}} \). The second has a diffusion coefficient \( D_{\text{free}} \) invariant with \( R \) and corresponds to tracers with a long range free diffusion. If \( D_{\text{free}} > D_{\text{conf}} \), domains are isolated with a long range diffusion taking place around them.

Note that, for relevance of the two-diffusion coefficient analysis, the weight of the free diffusion population should be in the same range as \( M_{\phi} \). All of the vrFRAP data have been analyzed following this procedure.

RESULTS

Functional Characterization of Stable Cell Lines—Four stable monoclonal cell lines were established to study the dynamics and confinement of CD4-eGFP and CCR5-eGFP receptors expressed alone or with their unlabeled counterparts. We first generated and sorted two cell lines expressing either CD4-eGFP or CCR5-eGFP. Further stable expression of wild-type CCR5 or CD4 (wtCCR5 or wtCD4, respectively) counterpart was generated, leading to two additional cell lines. The membrane expression of the receptors was verified by confocal microscopy. Fig. 2 shows that cells exhibited a homogeneous receptor distribution at the plasma membrane and that the fluorescence intensity originating from the intracellular compartments was negligible (<5%). For each stable cell line, with anti-CCR5 and anti-CD4 antibodies conjugated to phycoerythrin (1 mol of PhE/mol of IgG), the ratios between eGFP-tagged receptors and their wild-type counterparts were determined. Antibody solutions of equivalent concentrations were prepared based on absorbance and fluorescence measurements. Saturation curves were obtained by adding increasing amounts of antibody suspensions to cell solutions (Fig. 3). Fluorescence intensities were measured by flow cytometry. In both the CD4-eGFP + wtCCR5 and CCR5-eGFP + wtCD4 cell lines, the receptor/co-receptor ratio was about 1.

The functionality of the tagged receptors was previously checked (10). However, we ensured that the monoclonal cell lines sorted presented correctly folded and fully functional proteins. Fig. 4 presents the results of HIV-1 infection tests. Similar levels of viral replication were measured both in CD4-eGFP + wtCCR5 and CCR5-eGFP + wtCD4 cell lines. Furthermore, we observed a strong decrease of virus entry and replication by TAK779, a CCR5-specific ligand. Thus, neither the T7 tag at the N terminus nor the eGFP at the C terminus of the receptors hindered the induction of virus infection.

Since a modification in its composition could influence the membrane organization, we verified that the protein, lipid, and cholesterol contents of each cell line were unchanged compared with the wild-type cells (data not shown). Furthermore, to verify that the ectopic expression of receptors had no effect on the membrane dynamic structure, we measured the diffusion of fluorescent lipids (dC12-PE-NBD) by vrFRAP experiments. Quite similarly for the wild-type and transfected cell lines, lipids were found to have a long range free diffusion with a diffusion coefficient \( D = 1.8 \pm 0.5 \text{ \mu m}^2/\text{s} \). An immobile fraction of 30 ± 5% was also observed, consistent with the results of previous studies on HEK293T cells (12).

Diffusion of the Receptors Expressed Alone—Prior to studying the behavior of proteins in the presence of their counterpart, we first analyzed the dynamic of the receptors expressed alone. Fig. 5 shows that, when expressed alone, CD4-eGFP and CCR5-eGFP receptors presented similar diffusion characteristics. In both cases, the mobile fraction \( M \) increased linearly with the bleached area radius up to \( R \sim L \), indicating a receptor confinement into 1-µm-sized domains. By performing a two-population analysis and corre-
CD4 Interacts Constitutively with Multiple CCR5

lated with the low values of $M_p$, we concluded that for both receptors, only one diffusing population was present. CD4-eGFP receptors were confined into domains with a mean radius of 1.7 μm with $D_{\text{conf}} = 0.52 \mu m^2/s$, and CCR5-eGFP co-receptors were confined into domains of about 1.6 μm with $D_{\text{conf}} = 0.45 \mu m^2/s$ (Table 1).

The immobile fractions were found equal to 30 and 40%, respectively.

**Diffusion of the Receptors Expressed in the Presence of Their Counterparts**—In order to study the influence of the wild-type counterparts on the dynamics of the tagged receptors, we performed FRAP experiments on CD4-eGFP + wtCCR5 and CCR5-eGFP + wtCD4 cell lines. Interestingly, two distinct types of behavior were observed, depending on the receptor studied.

Within the experimental error, we obtained similar results for the CD4-eGFP + wtCCR5 receptors and CD4-eGFP alone (Fig. 5A and Table 1). In both cases, there was only one diffusing population ($M_p \approx 0$), and the domain size, the diffusion coefficients, and the immobile fraction varied less than 10%.

On the contrary, the behavior of CCR5-eGFP expressed alone was markedly modified by the presence of wtCD4 (Fig. 5B). Indeed, in the presence of wtCD4, two CCR5-eGFP diffusing populations were observed. The $M_p$ increased to about 50%, in total agreement with the weight of the long range diffusion population. This population had a long range diffusion coefficient $D_{\text{free}} = 0.4 \mu m^2/s$. A second population of CCR5-eGFP receptors remained confined in domains, but the presence of wtCD4 led to different diffusion parameters. The domain size decreased from 1.5 to 0.34 μm, and $D_{\text{conf}}$ decreased from 0.45 to 0.07 μm²/s (Table 1). Meanwhile, the immobile fraction, $100-M_p$, was decreased to a value of about 10%.

**Effects of sCD4 and gp120 on the Dynamics and Lateral Organization of the Receptors**—Our previous FRET experiments illustrated a disruption of the interactions between CD4 and CCR5 by the soluble part of CD4 (sCD4), whereas the viral gp120 protein strengthened the interaction (10). In order to further investigate their influence, we have performed FRAP experiments in the presence of saturating concentrations (100 nm) (18) of each of these molecules.

None of these proteins induced a change of the diffusion parameters of CD4-eGFP either expressed alone or in the presence of wtCCR5 (data not shown). Likewise, no

![Image](image1.png)

**FIGURE 4. Capability of CD4-eGFP + wtCCR5 (black bars) and CCR5-eGFP + wtCD4 (white bars) stable cell lines to be infected by the HIV.** HEK293T cells (105 cells) stably expressing tagged receptors with their wild-type counterparts on the dynamics of the tagged receptors, we performed FRAP experiments on CD4-eGFP + wtCCR5 and CCR5-eGFP + wtCD4 cell lines. Interestingly, two distinct types of behavior were observed, depending on the receptor studied.

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None of these proteins induced a change of the diffusion parameters of CD4-eGFP either expressed alone or in the presence of wtCCR5 (data not shown). Likewise, no

![Image](image2.png)

**FIGURE 5. Plots of the mobile fraction $M$ obtained in FRAP experiments versus the reciprocal of the radius of the bleached area ($R$) in different cell lines.** A, filled triangle, eGFP-CD4-expressing cells; open triangles, eGFP-CD4 + wtCCR5-expressing cells. B, eGFP-CCR5-expressing cells (filled circles); open circles, eGFP-CCR5 + wtCD4-expressing cells. Each symbol represents the average of a set of 50 – 80 experiments. The slopes of regression straight lines give us access to the size of the domains. The intercept with ordinates close to zero indicates the absence of a population of receptors with a long range diffusion (12, 17).

**TABLE 1**

Lateral diffusion parameters of CD4-eGFP and CCR5-eGFP receptors

| Experimental condition | $N$ | Domain size | $D_{\text{conf}}$ | $D_{\text{free}}$ | $M_p$ | $100-M_p$ |
|------------------------|-----|-------------|-------------------|------------------|-------|-----------|
| CD4-eGFP               | 440 | 1.7 ± 0.4   | 0.52 ± 0.29       | NA               | NA    | 10 ± 7  |
| CD4-eGFP + wtCCR5      | 320 | 1.6 ± 0.4   | 0.45 ± 0.29       | NA               | NA    | 17 ± 10 |
| CCR5-eGFP              | 400 | 1.5 ± 0.3   | 0.45 ± 0.17       | NA               | NA    | 15 ± 5  |
| CCR5-eGFP + wtCD4      | 280 | 0.34 ± 0.05 | 0.07 ± 0.10       | 0.40 ± 0.04      | ~50   | 51 ± 2  |
| CCR5-eGFP + wtCD4 + sCD4 | 100 | 1.1 ± 0.2   | 0.30 ± 0.14       | NA               | NA    | 18 ± 6  |
| CCR5-eGFP + wtCD4 + gp120 | 160 | 0.25 ± 0.04 | 0.06 ± 0.10       | 0.40 ± 0.04      | ~50   | 58 ± 3  |

* $NA$, nonapplicable because of the absence of a long range diffusing population.

* $100-M_p$ is the immobile fraction determined at the smallest radius (1.4 μm).

* $100-M_p$ is the immobile fraction determined at the extrapolation of the radius to $R \to 0$.

* The measurements for this condition are corrected according to the test condition of the day.
effect was detected on CCR5-eGFP expressed alone, but significant changes were observed in the presence of wtCD4. Indeed, after the addition of sCD4, the population of receptors with a long range diffusion disappeared, and the immobile fraction recovered to a value of 30%. Furthermore, a 3-fold increase of the domain size and a 4-fold increase of the diffusion coefficient of the confined receptors were observed (Table 1). This indicates that sCD4 disrupted CCR5-eGFP/wtCD4 interactions, rendering CCR5-eGFP behavior similar to when the protein was expressed alone.

However, the addition of the viral protein gp120 was not accompanied by significant modifications of the dynamics or the organization of receptors (only moderate changes of the diffusion parameters of confined CCR5 receptors were observed with wtCD4). The 20% decrease of the domain size and the absence of significant difference of the diffusion coefficient are consistent with a slight reinforcement of the interactions between CCR5-eGFP and wtCD4 by gp120. Conversely, no change on the diffusion behavior of CD4 was observed when gp120 was added to the CD4-eGFP + wtCCR5 cell line.

**DISCUSSION**

Viral entry mediated by the HIV-1 Env protein is a highly cooperative process that requires the presence of several receptors and coreceptors (3–5). Here we investigated the interaction and the dynamic of these molecules in order to have a better comprehension of the early events driving virus attachment to the host cell membrane.

**Diffusion Behavior of CD4-eGFP and CCR5-eGFP Proteins Expressed Alone**—The dynamic organizations of the receptors expressed alone were overall quite similar. First, our data suggest that a subset (about 30–40%) of CD4-eGFP and CCR5-eGFP receptors were immobile. This observation corroborates that of Steffens and Hope (19), who reported the existence of a similar immobile fraction for CD4 and CCR5. Since it was also reported in another work (20), the immobilization of a fraction of the CD4 receptors appears to be quite general. This could be attributed to interaction of this receptor with the cytoskeleton (20). The analysis of our vrFRAP experiments showed that both receptors exhibit a single population having a diffusion restricted into 1-µm-sized domains.

Whether the receptors expressed alone are in a monomeric state or not can be further deduced from our results. Since CD4 homo-oligomerization has been shown to occur via extracellular domains (23), the absence of change in the dynamics of CD4-eGFP expressed alone following the addition of sCD4 suggests that CD4 receptors are in a monomeric form. HEK293T cells being not lymphoid cells, they can be expected to express monomeric CD4 receptors. As a matter of fact, HEK293T cells compare more closely with resting T-cells, where CD4 is predominantly found as monomers, while the dimer expression increases markedly after T-cell stimulation (22). Consequently, the comparison between the diffusion coefficients of CD4 and CCR5 permits us to deduce that CCR5 is also probably in a monomeric state. The diffusion coefficient inside the domains of CCR5 appears to be slightly smaller by a factor of ~0.8 than that of CD4. Considering the respective sizes of the receptors (7 helices and 1 helix for CCR5 and CD4, respectively), this difference is in agreement with the value expected from the relationship between the size of a membrane protein and the lateral diffusion coefficient established by Saffman and Delbrück (21).

**Diffusion Characteristics of Receptors Expressed in the Presence of Their Wild-type Counterpart**—Although no effect of the co-expression of wtCCR5 on the CD4-eGFP diffusion behavior was detectable, wtCD4 expression strongly modified the dynamic organization of CCR5-eGFP. We suggest that a constitutive interaction between both receptors occurs, inducing a change in the receptor membrane dynamics. We propose that this cannot be evidenced by the analysis of CD4-eGFP diffusion, presumably due to the involvement of several CCR5 per CD4 in the interaction (this point will be developed further below).

The modifications of CCR5 receptor behavior in presence of wtCD4 are 2-fold. On one hand, confined CCR5-eGFP receptors have a diffusion restricted to smaller domains than those observed with CCR5-eGFP alone, and they have a decreased diffusion coefficient inside these domains. This is consistent with a CD4-CCR5 interaction inside these domains. On the other hand, wtCD4 counterpart expression induces the emergence of an additional mobile population with a long range free diffusion. The diffusion coefficient $D_{\text{free}}$ obtained for this second population is more rapid than the diffusion coefficient $D_{\text{conf}}$ obtained for confined receptors. Thus, according to the vrFRAP data analysis procedure explained above, we could characterize these domains as isolated. The confined receptors diffuse inside the domains, and the free receptors diffuse around them.

Finally, the CCR5-eGFP expressed alone presents an immobile fraction, about 30% of which tends to vanish in the presence of its wtCD4 counterpart. This supports further the existence of a basal interaction between CD4 and CCR5.

**Effect of sCD4 and gp120 on the Dynamics and the Lateral Organization of Receptors**—First, we investigated the influence of sCD4 on CCR5 and CD4 dynamic behavior. sCD4 has been previously shown to interact with CCR5 expressed alone (24) via its extracellular domain (10). However, unlike wtCD4, sCD4 does not modify the dynamics of CCR5. The transmembrane domain of CCR5 seems thus to be the major limiting factor of its diffusion. Indeed, in the absence of a dense glycocalix (data not shown), the extracellular domains of a transmembrane protein are in an aqueous medium and thus not expected to affect the diffusion, which tends to be rather dominated by the high viscosity of the membrane (21). The addition of sCD4 to the CCR5-eGFP + wtCD4 cell line strongly affected CCR5-eGFP dynamics, which became similar to the dynamic behavior of the receptor expressed alone. Thus, the interaction of sCD4 with CCR5 fully reversed the effect induced by wtCD4 expression, suggesting that sCD4 probably competes with wtCD4 for binding to CCR5. These results confirm the previous work, suggesting a constitutive interaction of CCR5 with CD4 (2, 10). Interestingly, we provide evidence here that CCR5 associated with CD4 has a dynamic organization different from that of CCR5 alone.

We then investigated the influence of the gp120 viral protein on CD4-CCR5 interaction. Previously, we have shown by FRET that the viral gp120 protein strengthens CD4-CCR5 interactions (10). This observed increase of FRET could have resulted...
CD4 Interacts Constitutively with Multiple CCR5

either from a closer proximity of donors and acceptors or from a rise in the number of interacting receptors. In our vrFRAP experiments, the absence of change in the respective weights of the two populations is in favor of the first hypothesis; CD4-CCR5 interaction was maximal in the basal state, and the addition of gp120 brings closer the two receptors.

Stoichiometry of CD4-CCR5 Interaction—vrFRAP measures the average dynamic of diffusing molecules, and different populations can only be distinguished if they represent more than 10–15% of tracers. On one hand, our experiments demonstrate that wtCD4 interacts with CCR5-eGFP, modifying its lateral organization within the membrane. On the other hand, the expression of wtCCR5 does not induce any detectable change in the dynamics of CD4-eGFP, even in the presence of gp120. The most straightforward explanation of this discrepancy is that less than 15% of CD4 are involved in a CD4-CCR5 interaction. Since the two receptors are expressed in a 1:1 molar ratio, one CD4 protein must be interacting with multiple CCR5 proteins. A rough estimation gives a ratio of 1 CD4 molecule interacting with at least 5 CCR5, consistent with data indicating that the assembly of 4–6 CCR5 around the virus is required for infection (4). In addition, according to the suggested preferential use by HIV of monomeric CD4 to gain entry into target cells (25), the receptors probably form complexes composed of multiple CCR5 for a single CD4.

Dynamics of Receptors and Kinetics of Interactions—Since the conformational changes of gp120 induced by the binding of CD4 are reversible (18), the gp120-CD4 complex must encounter CCR5 before gp120 dissociates from CD4. The lifetime of the CD4-gp120 interaction has been recently determined to be equal to about 0.2 s (11). Based on the value of the diffusion coefficient of CD4-eGFP, the largest area that the CD4-gp120 complex can explore within this time is 0.1 μm². Assuming that T cells have a radius of ~5.5 μm and express 10,000 CCR5 receptors homogeneously distributed at the cell surface (26), the CD4-gp120 complex would thus be likely to encounter only one CCR5 before disrupting. This would not allow infection to occur and reinforces our proposition that the receptors are compartmentalized within the membrane.

Conclusions—A microcompartmentalization of CD4 and CCR5 receptors into domains of ~350 nm was demonstrated by our vrFRAP analysis of the diffusion of the receptors at the surface of living cells. As demonstrated by the comparison with the behavior of the receptors expressed alone, this domain organization results from constitutive interactions between CD4 and CCR5. Moreover, we could establish the specificity of these interactions and determine their stoichiometry of about 1 CD4 for 5 CCR5. Therefore, an organization of the HIV co-receptors precedes the attachment of the virus at the cell surface that allows the accomplishment of the infection process. Indeed, the compartmentalization of CD4 would favor the binding of the virus to multiple CD4 (3, 5), and the stoichiometry of the CD4/CCR5 interaction would facilitate the subsequent multiple interactions with CCR5 (4).

Thus, the vrFRAP approach provided valuable information for unraveling the mechanisms of the early step of infection. While being consistent with our previous FRET study, we could here go one step further in the description of the dynamic organization of the receptors. Now the understanding of the origin and regulation mode of the compartmentalization remains challenging issues that single particle tracking experiments, currently under progress in our laboratory, should enable us to take up.

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