Mobility of the Human Immunodeficiency Virus (HIV) Receptor CD4 and Coreceptor CCR5 in Living Cells: Implications for HIV Fusion and Entry Events

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The sequence of events leading to human immunodeficiency virus fusion and entry likely involves the recruitment of multiple receptor and coreceptor proteins to a specific complex by the viral envelope. Using fluorescence recovery after photobleaching technology, we find that both CD4 and CCR5 are mobile in the cell membrane. Interestingly, our findings also suggest that the seven-span transmembrane coreceptor is significantly more mobile than CD4 and requires membrane cholesterol for mobility.

The events leading to human immunodeficiency virus (HIV) fusion and entry require cooperative interactions between the receptor, coreceptor, and viral envelope proteins. These interactions are dependent on the concentration and distribution of receptor and coreceptor molecules on the cell surface (3, 6, 15). However, much of this process is still unexplained. Previous studies have demonstrated the importance of actin and lipid rafts in entry events, but the mechanism of their involvement is unclear (5, 8, 9, 17). Recent observations by our laboratory and others suggest that the receptor and coreceptor have restricted localization in the cell membrane (16). This raises the question of how these proteins are recruited to virions to facilitate fusion (16). It is becoming clear that the interactions between the HIV envelope and the receptor and coreceptor take place in a multistep process. The first event is the interaction between gp120 and CD4. This interaction appears to facilitate conformational changes in gp120, which allow interaction with the coreceptor. Coreceptor binding is then believed to trigger extensive changes in gp41, leading to membrane fusion. Further studies suggest that multiple envelopes on the virion need to be engaged to facilitate fusion. For instance, observations suggest that the binding of multiple CD4s to each envelope trimer is required (7). The same appears to be true for the coreceptors, since Kabat's laboratory (6) found a nonlinear relationship between coreceptor expression levels and infection. These studies suggest that the cooperative interaction of virion envelope proteins with multiple CCR5 molecules is required to facilitate fusion. Another study focused on the kinetics of fusion events found that a 15- to 20-min time period is required after initial interaction for cell-cell fusion mediated by the HIV envelope to take place (10). The investigators found that if the cells are initially allowed to interact at 23°C before shifting to 37°C, no lag period is required. This observation suggests that a time-dependent step is required to develop the interaction necessary for fusion (10).

Together, these studies suggest that the proper engagement of HIV envelope requires the cooperative interaction of multiple CD4 and coreceptor molecules for fusion to take place.

The need for the engagement of multiple receptors and coreceptors to facilitate fusion begs the question of how HIV can interact with multiple cell surface molecules, leading to fusion. Recently it has been suggested that the receptor and coreceptor exist in microclusters in microvilli on the cell surface (15). Therefore, to a certain extent, this nonrandom distribution of molecules on the cell surface suggests that the initial localization of the receptor and coreceptor may facilitate cooperative interactions, as long as virions bind specifically at the site where the receptor and coreceptor are already concentrated. Alternatively, HIV may be able to move on the cell surface until it reaches a region of high receptor and coreceptor density. Another reasonable model is that CD4 and the coreceptor are recruited to the site of virion binding to the cell surface to facilitate formation of a fusion-competent complex. To gain a better understanding of interactions between HIV and its receptor and coreceptor, a better knowledge of the mobility of these cell surface proteins is required.

In order to gain insight into how CD4 and the coreceptor move on the cell surface, studies utilizing confocal microscopy and fluorescence recovery after photobleaching (FRAP) technology were conducted to directly monitor the lateral mobility of receptor and coreceptor in the cell membrane (14). Briefly, the confocal laser is projected on a small region of the cell of interest that stably expresses a fluorescent molecule. The region is exposed with maximal laser intensity until the fluorescent signal in the targeted region is irreversibly photobleached. After bleaching, the cells are imaged over time, and the recovery of fluorescent signal is determined at each time point. Recovery of fluorescence is due to the diffusion of unbleached fluorescent protein into the bleached region from surrounding areas (Fig. 1). The fluorescence intensity is then plotted over time and adjusted for overall photobleaching of the field scanned by the laser to determine the diffusional mobility of the protein of interest within the cell.

Three important aspects of the data are considered during analysis. First, the extent to which a fluorescent protein is initially photobleached provides information about the mobil-

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ity of that protein. It is possible for proteins to move during the process of photobleaching. Proteins that move faster in the membrane will be more difficult to bleach, since a rapidly moving protein would be able to diffuse from nonbleached areas of the membrane into the bleaching area while the laser is still on. Therefore, even these initial data are very informative. Second, the time required to recover 50% of the fluorescent signal is determined and utilized for comparative purposes. Finally, the overall extent of recovery of the fluorescent signal is considered. Proteins that move faster in the membrane will recover to a greater extent during the time that recovery is monitored. If the recovery plateaus before 100% recovery, it reveals the presence of a subset of the protein that is immobile on the cell surface. The presence of an immobile fraction will prevent 100% recovery because bleached proteins will occupy sites of protein binding that will not be replaced with nonbleached proteins during the time of analysis.

Key to these studies was the use of fluorescent protein constructs, including fusion proteins linking CD4 to yellow fluorescent protein (YFP) and CCR5 to green fluorescent protein (GFP). Validation and characterization of these proteins has been described previously in great detail (1, 16). The 293T, HeLa, and HOS cell lines were grown in Dulbecco's modified Eagle's growth medium (BioWhittaker, Walkerville, Md.) containing 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. 293T and HeLa cells were transiently transfected by using Effectene transfection reagent (QIAGEN), and expression was monitored 48 h later by fluorescence microscopy. Stable cell lines expressing either CD4-YFP or CCR5-GFP were utilized in addition to transiently transfected cells for these experiments. CHO 745 cells stably expressing cyclin T and CD4-YFP were grown in Isocove's medium containing 1 mg of blasticidin (Sigma)/ml in addition to the supplements described above. CD4-YFP- or CCR5-GFP-expressing cells were transferred to Delta T microscopy observation dishes (Biotechs, Butler, Pa.) for imaging. All experiments were conducted at 37°C, using a Zeiss LSM510 confocal laser scanning microscope optimized for live cell studies.

Experiments for CD4 and CCR5 were conducted on the same day under the same FRAP conditions, and results are adjusted to correct for photobleaching that occurred over the course of the experiment due to laser scanning at each time point. This adjustment is achieved by monitoring the fluorescent signal from cells in the same field of view as the cells being bleached. Although these cells do not receive the full intensity of the laser received by the cells being bleached, they are exposed during the scanning process of the entire field. This leads to a decline in the fluorescent signal, or photobleaching. Multiple regions of interest are monitored on cells receiving or not receiving bleaching treatment. Fluorescence intensity val-

FIG. 1. CD4 is mobile in the cell membrane. CHO 745 cells stably expressing cyclin T and CD4-YFP were subjected to FRAP analysis. The top panel depicts cells prior to photobleaching, with the area to be photobleached shown in a white box. After initial bleaching of the boxed area, the bleached area is revealed in the center panel. Recovery of fluorescence was monitored over time, and the final image is shown in the bottom panel (recovery time, 4 min).

FIG. 2. Mobility of CD4 and CCR5. (A) CCR5 is more mobile than CD4 in the cell membrane. CHO 745 cells stably expressing cyclin T and CD4-YFP and HOS cells stably expressing CCR5-GFP were subjected to similar FRAP conditions, and fluorescence recovery was monitored over time. The graph indicates individual representative experiments conducted on the same day with the same laser intensity. CCR5 is more difficult to bleach than CD4 and recovers to a greater extent. All results are normalized to those for other cells in the field of view for overall photobleaching that occurred as a result of exposure of the field to laser scanning during the monitoring of fluorescence recovery. (B) CD4 has an immobile fraction. CHO 745 cells stably expressing cyclin T and CD4-YFP were subjected to two rounds of bleaching by the confocal laser. The fluorescent signal was monitored and allowed to recover for the indicated amount of time between bleaching sessions. Data shown are from a representative experiment that was repeated on different days at different times. The CD4 signal recovered only to approximately 80% of the initial signal after the first bleaching. The immobile fraction of CD4 was bleached at this time. After a second round of bleaching, the CD4 signal recovered to approximately 100% of the fluorescent signal detected before the second round of bleaching.

ues are converted to a percentage of the value at time zero, and the average of the intensity values for each region of interest is calculated for each time point. Bleached-cell values are then adjusted according to the value calculated for unbleached cells. Results observed were confirmed with other cell types transiently transfected with the CD4-YFP and CCR5-GFP constructs (data not shown).

Initial studies focused on determining the mobility of the CD4 and CCR5 proteins in the cell membrane. Using HOS cells stably expressing the CCR5-GFP molecule, we determined that CCR5 is highly mobile in the cell membrane (Fig.

2A). Other experiments utilizing CHO cells expressing the CD4-YFP molecule demonstrated that CD4 is also mobile on the cell surface. Although our data suggest that both CD4 and CCR5 are mobile in the membrane, a subset of CD4 is immobile in its localization. Quantitation of CD4 recovery revealed that fluorescently labeled CD4 recovered only to 80% of the initial signal, suggesting the presence of an immobile fraction of CD4. This is consistent with a recent publication by Carpentier and colleagues, who also demonstrated that CD4 has an immobile fraction (4) that can be influenced by the expression of the tyrosine kinase Lck. Increased expression of Lck increased the percentage of the immobile phase. In the studies presented here, there is no Lck present in the CHO cells. The time to recover 50% of the CD4 signal is approximately 90 s. In contrast, GFP-labeled CCR5 consistently recovers to almost 100% of the original signal in the same amount of time (Fig. 2A). Together with our finding that CCR5-GFP is more difficult to bleach than labeled CD4, our data suggest that although CD4 and CCR5 have similar rates of diffusion, CD4 has a significant immobile fraction. These experiments have been repeated many times with similar results. Representative examples are shown in Fig. 2A.

Additional experiments were performed to further investigate the potential presence of an immobile fraction of CD4. Cells expressing the fluorescently labeled construct of CD4 were initially subjected to the same FRAP conditions as described above. Once the first cycle of bleaching and recovery was completed, the same cells were subjected to a second round of bleaching at the same regions of interest in the cell. As shown in Fig. 2B, CD4 recovered completely after the second round of bleaching. The observed recovery of CD4 in the rebleach was greater because the immobile fraction of CD4 present in the region of interest had already been bleached in the first round. Therefore, the immobile fraction does not contribute any fluorescence to the signal measured before the second bleaching. The complete recovery of CD4 after the second photobleaching demonstrates that a subset of CD4 exists in an immobile phase in the cell membrane.

A number of studies have suggested that the presence of CD4 and/or coreceptor in rafts may play a role in recruitment of these proteins (9, 13). Because cholesterol depletion has previously been shown to inhibit the ability of gp120 to induce CD4 and coreceptor colocalization (8), we determined the effect of the cholesterol-depleting compound methyl- β -cyclodextrin on CD4 and CCR5 mobility by utilizing the FRAP technique. Cells were incubated in the presence or absence of 10 mM methyl- β -cyclodextrin (Sigma) in Dulbecco's modified Eagle's culture medium supplemented with 10% delipidated fetal bovine serum for 1 h at 37°C. Cultured cells were then washed once with phosphate-buffered saline, and medium was replenished and subjected to FRAP analysis.

After methyl- β -cyclodextrin depletion of cholesterol from the membrane of HOS cells expressing fluorescently labeled CCR5, we observed very little recovery of the fluorescent signal in bleached areas in comparison with untreated controls (Fig. 3 and 4A). This suggests that cholesterol depletion renders GFP-labeled CCR5 immobile in the plasma membrane. Figure 3 shows representative examples of cells expressing CCR5-GFP before and after cholesterol depletion and FRAP. Even when an extended period of 11 min was allowed for

FIG. 3. Cholesterol depletion alters the mobility of CCR5. HOS cells stably expressing the CCR5-GFP construct were either treated with 10 mM methyl- β -cyclodextrin for 1 h at 37°C (bottom group) or left untreated (top group) and subjected to FRAP. Boxed regions indicate areas of photobleaching. Panels on the left depict cells prior to FRAP. Panels in center depict cells immediately after photobleaching. Right panels show cells after 11 min of recovery.

recovery, cholesterol depletion impaired CCR5-GFP mobility. Results shown in Fig. 4A are an average from two experiments conducted on the same day under the same FRAP conditions. This observation was reversible, since replenishment of cultures with cholesterol-loaded cyclodextrin allowed the recovery of the signal in bleached areas, reflecting a restoration in normal mobility (data not shown). These observations suggest that cholesterol plays a direct or indirect role in the mobility of the CCR5 coreceptor in the cell membrane. When CHO cells expressing CD4-YFP were subjected to similar conditions, we found that the mobility of CD4 did not appear to be significantly altered in the absence of cholesterol (Fig. 4B).

Our data suggest that the localization and mobility of the HIV receptor CD4 and the coreceptor CCR5 are both regulated and different. This finding may reveal one reason why HIV evolved to use the chemokine receptors as the coreceptors for infection. Proposed models of HIV entry describe a conformational change in the viral envelope protein after binding to CD4. This change exposes a highly conserved region adjacent to the V3 loop implicated in coreceptor binding. Extensive exposure of this region would increase viral susceptibility to immune recognition and the generation of neutralizing antibodies. However, a rapidly moving membrane protein that would quickly interact with and mask the V3 loop region would

be an appropriate target for binding. Consistent with this model, this is precisely the behavior that we have observed for CCR5 in our experiments. Thus, our finding that a subset of CD4 is relatively rigid in its localization and that CCR5 is highly mobile in the membrane is not surprising and thus may explain how this chemokine receptor gained a role in viral binding and entry events.

Consistent with previous studies, our findings also suggest that membrane cholesterol may play an important role in HIV entry (8, 13, 17). We observed that cholesterol depletion decreases the mobility of CCR5 but has no effect on the mobility of CD4. Current models of HIV fusion suggest that multiple receptor and coreceptor proteins must be recruited to one location in order for fusion pore formation to occur (3, 6). A recent study by Platt et al. (12) demonstrated that the concentrations of CD4 and CCR5 required for infection were interdependent and the need for each increased when only a limited amount of the other was available. Another study by Viard et al. (17) concluded that a lack of cholesterol in target cell membranes prevented HIV envelope from engaging coreceptor clusters when concentrations of the receptors were lower. These studies and others suggest the need for movement of the coreceptor in the membrane (10). Our observations demonstrate that this type of recruitment of the coreceptor can in-

FIG. 4. Differential mobilities of CD4 and CCR5 after cholesterol depletion. (A) Cholesterol depletion decreases the mobility of CCR5. HOS cells expressing fluorescently labeled CCR5 were either treated with 10 mM methyl- β -cyclodextrin for 1 h at 37°C (squares) or left untreated (diamonds) and subjected to FRAP analysis. Results from representative experiments are shown over time. Each line represents an average of two experiments conducted under similar conditions on the same day. Error bars indicate standard deviations. Cells treated with cyclodextrin recover at a much slower rate and to a lesser extent than untreated cells. All results are normalized for overall photobleaching that occurred as a result of exposure of the field to laser scanning during the monitoring of fluorescence recovery. (B) Cholesterol depletion has no effect on CD4 mobility. CHO 745 cells stably expressing cyclin T and CD4-YFP were either treated with 10 mM methyl- β -cyclodextrin for 1 h at 37°C (squares) or left untreated (diamonds) and subjected to FRAP analysis. Each line represents the average for two experiments conducted on the same day under similar conditions. Error bars indicate standard deviations. No significant differences were observed in the rate or extent of recovery of fluorescence between treated and untreated cells. All results are normalized for overall photobleaching that occurred as a result of exposure of the field to laser scanning during the monitoring of fluorescence recovery.

deed occur, and this type of movement in the membrane is impaired by cholesterol depletion. If the coreceptor CCR5 is unable to move in the cell membrane due to the absence of cholesterol, it cannot be recruited to a potential fusion site, thereby preventing fusion pore formation. Therefore, our findings may help to explain why cholesterol depletion inhibits HIV entry. A recent study by Nguyen and Taub has demonstrated that cholesterol is also important for other aspects of CCR5 functionality, including the maintenance of CCR5 conformation and the ability to bind chemotactic ligands (11). This

suggests that chemokine receptor function in chemotaxis may require receptor mobility.

Additional studies conducted by other groups suggest that CD4 and CCR5 are both localized within raft regions of the cell membrane (2, 13). However, our observations regarding the mobility patterns of CD4 and CCR5 suggest that there must be some difference between the localization or association of these two proteins with raft regions. Still other groups have suggested that a constitutive association exists between CD4 and chemokine receptors in stable complexes in the plasma membrane (18). Contrary to these findings, our observations and previous studies (16) demonstrate that CD4 and CCR5 can move independent of one another in the membrane and have the potential to do so during fusion pore formation.

In summary, the areas of the cell membrane where the HIV receptor CD4 and coreceptor CCR5 localize are dynamic. Our studies allowing visualization of fluorescent fusion proteins using high-resolution deconvolution microscopy and FRAP technology demonstrated that although these proteins tend to accumulate in actin-dependent, ezrin-enriched cellular structures (16), they are mobile within those structures. Thus, the close proximity resulting from the preferential localization of both CD4 and CCR5 in actin-dependent structures and at sites of cell-cell contact may have broad implications for how HIV interacts with the receptor and coreceptor. However, it may be the mobility of these proteins, specifically the coreceptor CCR5, in the cell membrane that is crucial for the formation of a potential fusion pore.

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