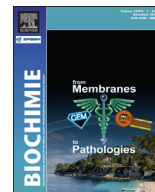




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Mini-review

Membrane organization of virus and target cell plays a role in HIV entry



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ABSTRACT

The initial steps of the Human Immunodeficiency Virus (HIV) replication cycle play a crucial role that arbitrates viral tropism and infection efficiency. Before the release of its genome into the host cell cytoplasm, viruses operate a complex sequence of events that take place at the plasma membrane of the target cell. The first step is the binding of the HIV protein envelope (Env) to the cellular receptor CD4. This triggers conformational changes of the gp120 viral protein that allow its interaction with a co-receptor that can be either CCR5 or CXCR4, defining the tropism of the virus entering the cell. This sequential interaction finally drives the fusion of the viral and host cell membrane or to the endocytosis of the viruses. Here, we discuss how the membrane composition and organization of both the virus and the target cell can affect these steps and thus influence the capability of the viruses to infect cells.

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1. The entry of HIV virus in the target cell follows a complex sketch

The entry of the virus into the cell can be decomposed in three main successive steps: 1/the interactions of the viruses with their specific receptors CD4, 2/the interactions of the viruses with co-receptors (CCR5 or CXCR4) and 3 or 3'/either the fusion of the membrane of the virus with the one of the infected cell or the endocytosis of the virus (Fig. 1). The efficiency of each of these steps is influenced by the membrane protein and lipid composition and by their lateral organization and dynamics at the surface of the target cell.

1.1. Binding to CD4 receptors

It has been shown that HIV attachment to immune cells first occurs *via* non-specific adhesion molecules that restrict the virus envelope in vicinity of more specific receptors [1,2]. CD4 is the principal and mandatory receptor. Upon binding to CD4 (step1), the viral gp120 protein undergoes a conformational change allowing

additional interactions with a co-receptor (step 2). The main parameter that can affect the efficiency of this first step is the density of CD4 receptors that are present at the surface of the target cell [3–5]. This amount varies a lot from cell to cell type – in macrophages CD4 expression levels are 10–20 fold lower than what they are in CD4⁺ T cells – but also depends on the activation state of immune cells [6,7]. For these reasons, there is a huge variability of infection efficacy depending both on the virus strain and the cell type which is infected, rendering difficult the interpretation and the comparisons of experiments carried out in different studies.

Beyond the expression level of CD4, a second factor that has to be taken into account is the lateral organization of the proteins. It is now well known that the lateral organization of proteins in the plasma membrane of the cells is heterogeneous [8,9] and might depend on lipid composition [10]. In this context, many laboratories have observed that CD4 proteins are not randomly distributed at the surface of the plasma membrane of cells but rather confined into domains [11–13]. Such a clustering may promote HIV binding since viruses could engage several interactions with multiple CD4 confined in a small area.

CD4 has been shown to be partially located into lipid rafts [14]. Lipid rafts microdomains result from the preferential association between sphingolipids, saturated lipids, sterols and specific proteins in the external leaflet of the plasma membrane. These microdomains are found in a liquid-ordered state (Lo) that renders them resistant to solubilization by detergents at low temperature

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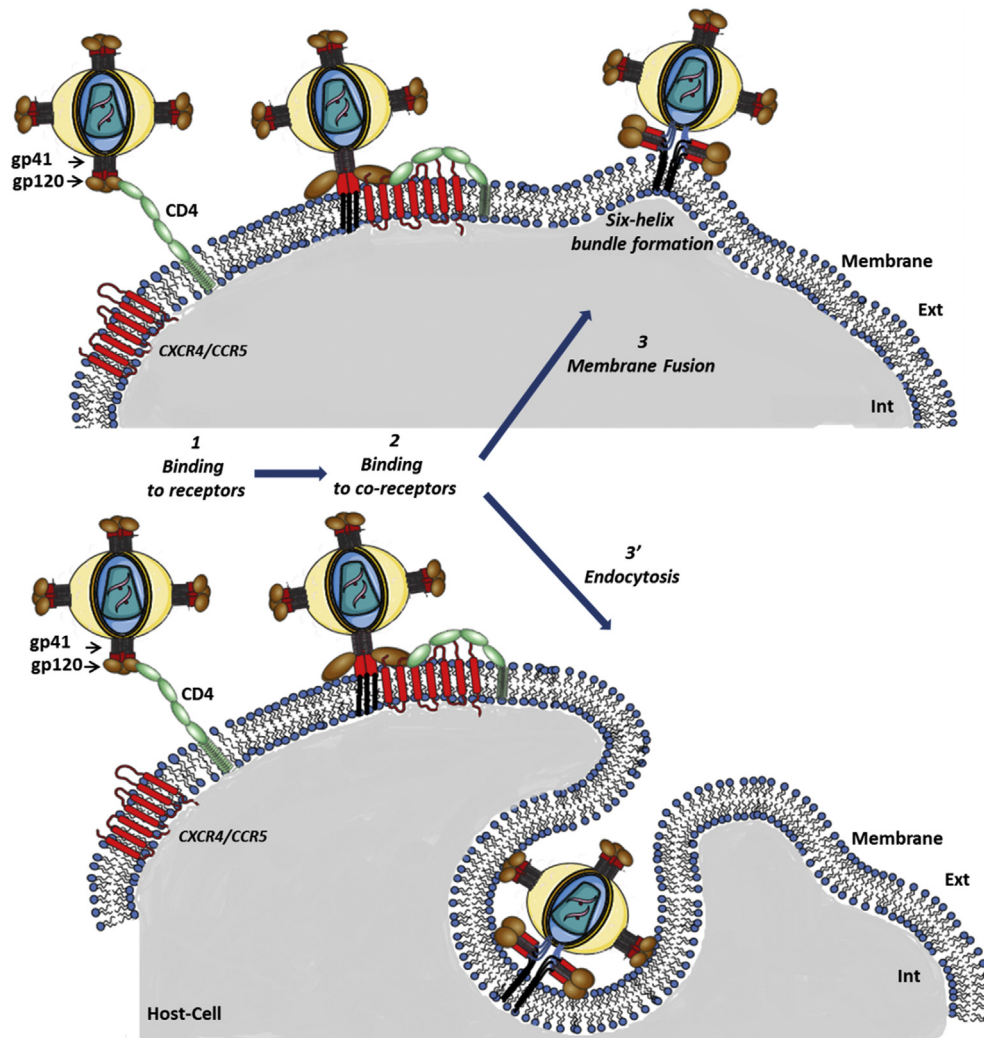


Fig. 1. Schematic view of HIV-1 early steps of infection. The upper part represents the HIV-1 virus envelope. The bottom part corresponds to the infected immune cell (T-lymphocyte, macrophage or dendritic cell). 1- **Binding of CD4 to gp120** leads to exposure of a co-receptor binding site in gp120. 2- **Binding of gp120 to co-receptor**: depending on virus tropism co-receptor can be either CCR5 (R5 tropism) or CXCR4 (X4 tropism). Co-receptor binding triggers new conformational changes of gp120, exposing gp41 protein that inserts into the membrane of host cell. 3- **Fusion**: gp41 protein bends back on itself, forming a six helix bundle that bring closer the viral and target cell membranes leading to their fusion. 3'- **Endocytosis**: an alternative pathway to fusion is the entry of virus into its cell host *via* endocytosis. Virus particle then can be degraded or they can fuse with vesicular membrane, releasing virus content into the cytoplasm of the cell.

[15–17]. There is a growing amount of evidence that the presence of cholesterol in the target cell is essential for the infection by many viruses. Ebola virus [18], herpes simplex [19,20] or murine coronavirus [21] all require cholesterol in the target cell membrane. A long controversy started at the beginning of the millennium to know whether HIV preferentially binds to CD4 proteins that are confined into lipid rafts or not. The putative role of lipid rafts first came from the observation that cholesterol depletion impaired HIV infection [22–24]. Similar results have also been obtained by using glucosylceramide synthase inhibitors that blocks glycosphingolipid biosynthesis [25]. Nevertheless, these approaches present two drawbacks. First, these treatments are very stressful for the cells and one cannot be sure that the observed effects are the result of the sole decrease in cholesterol content. Second, it does not reveal at which step lipid rafts might be involved in the infection process (receptor or co-receptor binding, fusion...). To go further in the comprehension of the role of lipid rafts in HIV infection, detergent extractions and optical microscopy studies have been used and produced contradictory results. As an example, one study showed that a CD4 mutant excluded from lipid raft domains membranes did

not allow efficient HIV entry [26], whereas a similar study, using alternative mutants that also prevent CD4 association with lipid rafts, established that CD4 excluded from lipid rafts do support HIV entry [14]. These two studies have been carried out on different cell types (HEK and T cells, respectively). These cell lines very likely have different lipid composition and organization. This might explain the discrepancies in the results that have been described above. This also enlightens the crucial role of membrane composition and organization on the mechanisms of infection process. Apparent contradictions also arise from works published by the same authors that successively showed that HIV uses lipid raft-co-localized CD4 for productive entry into cells [24] and that CD4 receptor localized to non-raft membrane microdomains supports HIV-1 entry [27]. One hypothesis, that renders these puzzling observations compatible, is to take into consideration that these studies are probing different steps of the infection process (i.e. binding to CD4 receptor, binding to co-receptor or fusion). Our proposal is that the lipid raft localization of CD4 is not required for virus binding while the following steps are dependent on the presence of lipid rafts. This hypothesis is reinforced by the

observation that CD4 lateral mobility affects HIV-1 envelope glycoprotein mediated fusion [28]. In other words, once viruses are bound to the cell surface they might migrate to lipid raft domains where the infection can be completed. Furthermore, this model is in agreement with the observation that murine leukemia virus “surfing” at the surface of infected cells precedes entry [29]. This shows that the different steps of HIV infection do not occur at the same place rendering necessary the development of dynamic approaches in order to study the time-course of infection process.

1.2. Binding to co-receptors

The major co-receptors of HIV-1 are the chemokine receptors CCR5 and CXCR4. The use of the co-receptors defines the viral tropism of the strain that will infect the cell. Viruses using CCR5 as a co-receptor are called R5, those using CXCR4 are named X4 while those that use both are termed R5X4 [30,31]. R5 viruses prevail during the chronic phase of the infection while the emergence, after several years and for half the patients, of variants capable of using CXCR4 is associated to the rapid progress towards a pathologic state [32]. The mechanisms by which X4 viruses emerge and precipitate disease progression are still unknown. Different studies have proposed that the infection process requires the attachment of several gp120 envelope trimers to multiple CD4 and multiple co-receptors [33–36]. Considering the weakness of CD4-gp120 interaction [2], the viruses should have a very short time (less than 0.2 s) to encounter their co-receptors once bound to CD4 [37,38]. Virus entry might thus not only depend on the local membrane density of CD4 but also on that of chemokine receptors. This local density is expected to be favored by the sequestration of CCR5 and/or CXCR4 with CD4 into delimited membrane domains. This is further supported by high-resolution electron microscopy experiments showing that, CCR5, CXCR4 and CD4 are clustered on microvilli of human macrophages and T cells [13].

1.2.1. CCR5 co-receptor

Co-immunoprecipitation studies have shown a constitutive association between CD4 and CCR5 at the surface of T cells, macrophages and monocytes [39]. Similar studies performed on HEK cells showed the opposite [40]. A constitutive interaction has later been confirmed by FRET experiments that also revealed that this interaction takes place outside of lipid raft domains and is reinforced by the viral gp120 protein [41]. Another clue of CD4-CCR5 interaction came from the study of the membrane dynamics of these receptors. It has first been observed that CD4 and CCR5 presented different mobility [42]. More interestingly, we have shown that the presence of CD4 strongly affects the dynamics of CCR5. Indeed, when CCR5 was expressed alone in HEK 293T cells only one diffusing population confined into large domains was detected. This behavior was markedly modified in the presence of CD4 since two diffusing populations of CCR5 were then observed, leading to the conclusion that the interaction of one CD4 with several CCR5 leads to their confinement into smaller membrane domains than in the absence of CD4 [11]. These confinement zones that concentrate both CD4 and CCR5 might constitute platforms that would facilitate the binding (and/or consecutive fusion) of HIV to its target cell.

1.2.2. CXCR4 co-receptor

Because of the prevalence of R5 virus strain in primo-infections, most of studies concern CCR5 receptor and, comparatively, only few studies have been carried out on CXCR4 protein organization within the membrane of immune cells while many works concerns clinical investigations [43]. As for CCR5, a co-immunoprecipitation study has shown a constitutive association between CD4 and CXCR4 [40]. However, this study has been performed in HEK 293T cells

transiently expressing CD4 with CXCR4 or CCR5 that gave contradictory results regarding CCR5 [39] as mentioned in the above CCR5 co-receptor chapter. More recent publications, respectively based on the use of cyclodextrin and ceramide modulators, have also shown that CXCR4 proteins are partially localized into lipid rafts domains and that this localization into lipid rafts is required for efficient infection [44,45].

Even though literature can be contradictory in some points, it seems to be clear that receptor and co-receptors of HIV virus are confined into domains that could act as ports of entry for HIV viruses. This is for example illustrated by the fact that statins inhibit both R5 and X4 virus infection by down-regulating the activity of Rho GTPases which is expected to impede the clustering of host lipid raft-associated receptors [48]. The existence of different and independent confinement zones might also give an explanation for R5 prevalence at the expense of X4. For instance one can hypothesize that CD4 is preferentially confined with CCR5 into microdomains different from those containing CXCR4 [22]. This hypothesis is in agreement with the observation that reducing the expression of CD4 decreases the susceptibility of human cells to infection by X4 viruses [46]. Furthermore, it has been shown that lowering CCR5 expression level with molecules modulating cholesterol content (lovastatin, mevastatin and simvastatin), favors the infection by X4 viruses [47].

Taken together these results raise the question of the lateral distribution and the stoichiometry of the different receptors at the surface of the target cell. Recently, Johnston and collaborators have developed affino cells in which the expression level of both CD4 and CCR5 can be independently modulated [49]. Several strains stably expressing various amounts of CXCR4 have been generated; this allows obtaining any ratio of CD4, CCR5 and CXCR4. This tool provides a powerful method to characterize viral entry efficiency as a function of CD4 and CCR5 expression levels [50]. These affino cell lines also permitted to demonstrate that several CCR5 conformations (distinguished thanks to specific monoclonal antibodies), exhibiting different localization and G-protein association, might have implications in selective targeting of HIV-1 [51]. This system is also used in our lab to check whether the expression level of receptors and co-receptors can impact the dynamic of each other and the infection efficiency of various virus strains.

1.3. Fusion

Receptor binding and subsequent association with the co-receptor, lead to conformational changes of the viral trimeric gp120 protein. This induces the exposure of the hydrophobic gp41 peptide which inserts into the host cell membrane. This brings together the viral and host membranes. The gp41 protein then bends back on itself, forming a six helix bundle [52,53]. At this stage, the viral membrane and the target cell membrane have bypassed the repulsion forces and are close enough to spontaneously form the fusion pore [54,55].

The role of lipids in the modulation of this step has been extensively studied and the implication of lipid rafts is now admitted by most of researchers. It has first been shown on large unilamellar vesicles (LUV) that sphingomyelin and cholesterol promote gp41 surface aggregation and membrane restructuring [56]. Such an aggregation is thought to be important to reach a stoichiometry allowing the fusion to occur. Indeed it has been proposed that at least two envelop proteins have to bind CD4 and a co-receptor and that two fusion proteins of each trimer have to insert into the target cell to promote viral entry [57]. The gp41 protein presents an extracellular domain that contains the fusion peptide whose structure strongly depends on its lipid environment. Some authors have proposed that a small amino acid sequence

(LWYIK), within the gp41 protein, constitute a cholesterol-binding domain [58] which is important for HIV infectivity [59]. This sequence presents homology with the Cholesterol Recognition Amino-acid Consensus (CRAC) motifs described by Epanand [60]. Further works realized on model membranes confirmed that the ectodomain membrane proximal region of gp41 was able to bind to cholesterol membrane domains [61]. The binding to such a cholesterol-rich (>30 mol%) zone promotes β -sheet secondary structuration of the fusion peptide that deeply embeds into the host membrane. This deep insertion has been shown to favor fusogenicity [62–64]. Beyond these effects on insertion and structuration of the gp41 virus protein, cholesterol might play a role in the membrane fusion itself. The fusion involves the coalescence of viral and hosts cell membranes, accompanied by the mixing of their cytoplasm. This implies that lipids transiently adopt a non-lamellar organization as described by Chernomordik and Kozlov [65]. Such an organization, named “hemifusion stalk”, can be stabilized by inverted cone-shaped and/or by cone-shaped lipids, depending on their outer or inner leaflet location. From this point of view, it is interesting to note that HIV membrane is enriched in phosphoinositides [66] that can induce a positive curvature that favor the initiation of fusion while cholesterol might mediate membrane curvature of host cell during fusion [67]. It has to be noticed that this effect of cholesterol is not specific to HIV fusion process and has been observed in other processes such as nuclear membrane formation [68].

Other authors have shown that sphingomyelin plays a role in HIV infection. A study based on surface aggregation assays of rhodamine-labeled gp41 subunit demonstrated that sphingomyelin and cholesterol promoted gp41-aggregation [56]. The effect of sphingomyelinase has also been studied by extraction of rafts, virus binding assays and measurements of the dynamics of HIV receptors. The authors observed that sphingomyelinase treatments decreased CD4 lateral mobility and inhibited HIV fusion with the membrane of the target cell. They propose that these effects are due to the clustering of CD4 molecules that prevents co-receptor engagement and HIV fusion [69].

It has to be noticed that the fusion of the viral membrane and the plasma membrane of the target cell does not necessarily conduce to productive infection since the viral content can be degraded inside the target cell. Furthermore, lipid mixing of viral and cell membranes have been observed without release of the viral content inside the target cell [70,71]. This incomplete fusion is an argument in favor of an endocytotic pathway discussed in the following section.

1.4. Endocytosis

Although the mechanism of HIV fusion is now well documented (see part 3 of this manuscript), several lines of evidence suggest that HIV entry into their cell host can also occur *via* endocytosis [55,72–76]. This process has moreover been proposed to be predominant in HIV internalization into macrophages, endothelial cells or epithelial cells [31,77–80] and has also been shown to depend on the cell activation status [81]. This mini-review is focused on the role specifically played by lipids in this process, we invite the reader to refer to the recent review by Melikian for a more general discussion of endocytosis versus fusion in HIV entry [71].

Kinetic measurement of fusion performed at different temperatures and the use of specific inhibitors pointed out that HIV viruses enter the cells *via* endocytosis followed by fusion with endosomes [70]. This work also showed that inhibiting endocytosis allowed lipid mixing of viral and cell membrane without conducting to a complete fusion. Interestingly, the obtained results were

independent of virus tropism but kinetics appeared to depend on the target cell type. This might be due to differences in endosome trafficking, but it is tempting to propose that these variations might be due to differences in lipid composition of these cell lines. As an example, in similar experiments, Markosyan and collaborators have proposed that membrane tension might differ between cell types and affect pore formation and growth [82].

A study, based on cholesterol disruption of macrophage membranes, demonstrate that HIV entry into macrophages is dependent on lipid rafts [83]. This has later been confirmed by a study showing that endocytosis of HIV involves rafts [84]. The originality of this work comes from the fact it does not use pharmacological agents that may have non-specific effects. Thanks to genetically modified macrophages allowing manipulating the sub-cellular distribution of CD4, these authors have that the entry of HIV into macrophages is dependent on endocytosis through lipid raft containing CD4 [84].

Only few works have been carried out to specifically study the role of lipids in HIV endocytosis and it will be interesting to go further in this field.

2. Concluding remarks

One difficulty to understand HIV infection mechanism comes from the fact that different entry pathways exist and the importance of each being still a subject of controversy. Since most of HIV particles are degraded by the cells and only a small fraction of viruses establishes infection, the identification of productive entry pathway remains difficult.

However, whatever the mode of entry of viruses, many observations suggest that membrane composition and organization play important roles. Cholesterol, sphingomyelin and lipid raft domains seem to be of first importance not only in the infection process but also in virus prevalence [47]. Glycosphingolipids and gangliosides have also been reported to facilitate HIV infection such as galactosylceramides [85] or GM1 and GM3 [86,87]. These lipids might serve as HIV binding sites or regulate dynamic and clustering of CD4, CCR5 and CXCR4. This information has been recently reinforced by the fact that peptides conjugated to lipid comprising sphingomyelin backbones impair early and late HIV-1 membrane fusion [88].

In this context, the development of new original and non-invasive techniques should allow to discriminate the entry pathway of viruses and to decipher the molecular mechanisms involved in the infection process. Among them, the Single Particle Tracking technique is promising. It has been used to study the dynamics and the organization of HIV receptors and co-receptors, revealing the existence of subpopulation of CD4 with different behaviors in T-lymphocytes [12]. Tracking entire viruses consists in labeling viruses with fluorescent probes and recording their movements with a videomicroscope. This might be useful to study the time-course of infection process. This approach gives information regarding the percentage of aborted binding (i.e. virus that bind to CD4 without encountering co-receptors and finally unbind). It also permits to measure the time-lapse from binding to cell surface until virus and target cell membranes fusion. These two parameters give real time insights onto the efficiency of the infection process. This method will be useful to verify previously obtained data on the behavior of single proteins in a more physiological and relevant context. Such an approach has already been successfully used with influenza viruses [89,90] and might be extended to inactivated HIV viruses that keep their binding and fusion properties [91,92]. Combined with the use of drugs to modulate lipid membrane composition these techniques will permit to understand the mechanisms involved in infection and more specifically the role played by lipids in this process.

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