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How Do Cell-Free HIV Virions Avoid Infecting Dead-End Host Cells and Cell Fragments?

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Abstract

HIV faces the challenge of identifying and entering suitable host cells (i.e. activated and viable) among a wide array of receptor-positive but unsuitable targets. Lymph nodes contain resting cells, activated cells destined for apoptosis within 24 h, and cell fragments, all of which represent replicative dead ends. We postulate that 1) HIV virions have evolved the ability to probe the internal status of potential host cells from the external cell membrane by assessing the ability of cells to co-cap CD4 and chemokine receptors, and 2) the requirement for dual receptor binding in a concerted manner by three gp120 molecules is the molecular mechanism by which virions stochastically ensure high density co-capping of receptors. Cell-associated HIV accomplishes the same selective process by targeting cells capable of participating in immunological synapse formation. **(AIDS Reviews 2004;6:155-60)**

Key words

Apoptosis. HIV. Immunological synapse. Chemokine receptors.

The conundrum of dual receptor binding

In exploiting the full potential of its 10 K base-pair genome, the HIV demonstrates remarkable adaptability and efficiency in the face of an evolving immune response and a changing host-cell population. The entry requirement for two cell surface receptors, CD4 and a chemokine receptor (CKR), would appear to place an additional restrictive burden on this limited genome, and decrease the efficiency of entry into new cells. The most commonly proposed explanation for the dual receptor phenomenon is the protection it affords cryptic binding sites, thereby protecting them from neutralization[1]. However, this begs the question of neutralization at the penultimate CD4 binding site, which is not rendered any less vulnerable by the existence of cryptic CKR binding residues. We propose an alternative or additional complementary explanation for the evolution of dual binding, based on the survival advantages to HIV of entering healthy, activated CD4+ T-cells.

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The problem of unsuitable host cells

One feature of the HIV life cycle that has been studied from early in the epidemic is its preference for entry into, and replication within, activated cells. Engleman's group was among the first to suggest that the virus could bind, but would not efficiently enter resting T-cells[2]. Subsequent research revealed that resting T-cells can be infected *in vitro* and *in vivo*[3], but with a significantly longer time required for completion of reverse transcription *in vitro* (three days vs. hours) compared to activated cells[3]. Concomitant decay of partial and completely linear DNA prior to integration results in fewer than 15% of resting T-cells able to subsequently integrate and express HIV upon cell activation. Since resting cells express high levels of CD4 and varying levels of the appropriate chemokine receptors CCR5 and/or CXCR4[4,5,6], a question

arises at the outset of the HIV life cycle: "Can a potentially infectious virion avoid entering a resting host T-cell in which its chances of propagation are decreased compared to an activated cell?"

However, the issue of host-cell selection is even more fraught with risk for the virion, because entry into an activated host cell does not automatically solve the problem of host-cell suitability. The life-span of a normal activated T-cell following receptor stimulation is ~72 h (~1 day required for full activation, and ~48 h thereafter), due to the well-studied phenomenon of homeostatic activation-induced programmed cell death[7]. About 95% of normally activated T-cells are destined to undergo apoptosis within a few days of stimulation. Thus, at sites of immune system activation in the lymph nodes, gut and spleen, which are the primary loci of viral replication[8] and transmission, at least one quarter of activated cells are destined to die within 24 h. After several days of localized immune response (within a lymph node, for example), the great majority of activated T-cells may be destined for apoptosis within the next 24 h. This is critical for newly produced virions, because a full cycle of HIV replication, from binding to budding, requires a minimum of ~24 h under optimal conditions of cell activation[1,9]. Furthermore, activated and apoptotic cells release large amounts of plasma membrane in the form of blebbed vesicles, which may carry surface CD4 and CKRs[10-12].

In light of these considerations, the question posed above may be expanded: "How can HIV virions avoid irreversible fusion with resting host cells, activated host cells destined for imminent apoptosis, and cell fragments containing surface receptor molecules?" Because HIV within a given human host exists as a swarm, exhibiting constant mutation and rapid Darwinian evolution in response to selective pressures of immunity and niche availability, we postulate that successful HIV (i.e. virus circulating in infected individuals) will have evolved mechanisms for reducing irreversible entry into dead-end host cells or cell fragments.

Obstacles to cell free vs. cell-associated transmission

The most straightforward way for HIV to preferentially encounter healthy, newly activated CD4 T-cells is via cell association. Thus, virions within long-lived, infected antigen presenting cells (macrophages or dendritic cells) are likely to be transmitted cell-to-cell only when there has been intimate surface contact with another cell, typically a CD4+ T-cell, recently activated, most likely by cognate recognition of some antigen on the infected antigen presenting cell. Under these conditions, HIV is assured of entry into viable new host cells[13]. Similarly, formation of long-lived, syncytial giant cells, while probably not a major feature of R5 HIV infection *in vivo*, would ensure similar levels of intracellular activation. But what of virions released directly into the extracellular fluid, either by rupture of infected cells or non-lytic budding?

Cell-free virus could accomplish the selection of suitable host cells in two stages. The first would be the release of molecules along a gradient attracting only healthy activated cells. HIV gp120 does, in fact, have cell-activating properties, triggering Rac-1 GTPase and stimulating the actin filament network[14-17], as well as potent chemotactic properties for activated CD4+ T and dendritic cells[18]. It is likely that the release of free envelope from sites of intensive HIV replication recruits additional target cells to the immediate area. While isolated virions can shed gp120, the small number (~100) of trimers present on the virion[19] would rapidly result in loss of virion infectivity. In contrast, release of excess gp120 during localized virion bursts, could attract new host cells with sufficient anatomical precision to propagate a "founder effect"[20] with selective evolutionary significance.

Interrogating the potential host cell

Attracting activated host cells would not necessarily reduce the numbers of apoptotic cells and cell fragments in the immediate vicinity of budding virions. Therefore, a method of discrimination is needed, entailing sampling by the virion of the host cell's internal activation status and viability, from the relative safety of the external cell membrane. This mechanism requires that once a potential target cell is sampled and found wanting as a suitable host, the membrane-attached virions remain infectious on the surface of non-viable hosts, and capable of subsequent release and infection of more suitable targets. Both requirements are consistent with the structure of HIV. But how is the simultaneous sampling of potential host-cell activation and longevity accomplished by a simple virion? Any proposed mechanism must posit the ability of the HIV virion to detect transmembrane signals and responses related to cell viability. This may be considered the second stage of host-cell selection.

As a budding virus, HIV has two potential sources of surface molecules with which to probe the transmembrane response of target cells – viral genome encoded

envelope proteins, and host membrane proteins acquired during the budding process. The latter include many known ligands for signal transducing receptors, such as intercellular adhesion molecules and HLA, which could initiate signal transduction via cross-linking of cell surface receptors. HIV envelope gp120 has the capacity to cross-link CD4 and CKRs to initiate signal cascades[14-18]. In the case of gp120, signaling may be modulated by virion-bound anti-envelope Abs which could further cross-link surface bound envelope, or find purchase on target cell membranes via Fc receptors. The ability of the membrane bound virion to trigger one or more cell-signaling cascades could be the first part of the host-cell selection process.

Alternatively, virion surface proteins could bind to host-cell surface components already undergoing modulations indicative of intracellular conditions, either conducive or inhospitable to HIV. This would be particularly important for virions traveling through the circulation to sites distant from the foci from which they originated, as individual virions would be unlikely to trigger cell activation. An example of the former would be the coreceptor capping at the leading edge of migrating cells (see below). A theoretical example of the latter would be the ability – purely speculative – of HIV to detect the presence of external membrane phosphatidyl serine, a marker of apoptosis.

Whether triggered by virion binding, or ongoing at the time of binding, signals generated by the host cell must be interpretable by the externally bound virion as "go" or "no-go" for entry. Again the virion has, in theory, the same two distinct sources of surface molecules for this interpretive task: host membrane derived, and virally encoded. While limited virus membrane reorganization (of lipid rafts, for example) might be envisioned in response to engagement of some host-derived molecules, there is no evidence to suggest that viral membrane bound host-cell proteins can transduce signals back to the virion; associated inner-membrane proteins are not present in the virion, nor is the molecular machinery for responding to second signals. Additionally, experiments designed to assess the contribution of various cell-encoded ICAM molecules, such as LFA-1, to viral binding and fusion have revealed a modest ancillary contribution, but not a critical role[21].

By contrast, the viral envelope proteins, gp120 and gp41, are poised for rapid, concerted, entropy-driven structural changes that have dramatic impact on the function of host-virus membrane fusion. This membrane fusion is an irreversible step, and thus represents commitment of virus to the host cell in question. How do the HIV envelope proteins enable the virion to accurately interrogate the host cell's status? The answer may lie in the combination of the stoichiometry and the stochastic nature of virion binding and fusion. Biochemical and crystallographic data suggest that the HIV envelope proteins are arranged as trimers on the virion surface, the protruding gp120 molecules forming a protective cage around the hydrophobic fusion component gp41, which also exists as a trimer with a buried head that is released from the viral membrane at time of fusion, in a manner analogous to influenza virus hemagglutinin[1,22-25]. It is estimated that there are fewer than 100 trimers per virion[1,19], and it is likely that more than one trimer host-cell interaction is required for successful membrane fusion[6].

Stoichiometry dictates high receptor density

Recent studies using atomic force microscopy shed a somewhat different light on the surface arrangement of HIV molecules[19]. No evidence was found for symmetric trimeric association of gp120 molecules, or for sharply differentiated envelope "spikes". Rather, ~100 broad "tufts" per virion were distributed in varying patterns. The authors speculated that gp120 might be nonspecifically associated in "bouquets" of random size. Absence of strict trimeric envelope stoichiometry may account for the findings of Kim, et al. that CD4 binds to gp120 monomer in a 1:3 ratio rather than the expected 1:1, although these authors favor a different explanation of trimeric anti-cooperativity based on conformational hindrance[26]. By contrast, Kumann, et al.[27] and Doms' group[6] found evidence of cooperativity and estimated 3-6 gp120 – CD4 interactions required for successful initiation of fusion.

While the exact stoichiometry of gp120:gp41 at the virion surface may be unresolved, the preponderance of biochemical, immunochemical, and structural evidence strongly suggests that at least three members of the gp120 complex (i.e. one trimer) must engage their ligands simultaneously for fusion to occur[6,22-25,27-31]. Theoretical considerations also strongly support the need to create a (minimally) trimeric cage of hydrophobicity, allowing the gp41 buried heads to traverse the inter-membrane space in a concerted fashion, to initiate the fusion event. Therefore, for the sake of this discussion, we will use the simplest and most parsimonious stoichiometry of trimers as the minimal struc-

ture that must engage host-cell receptors. The chance of this occurring is further reduced by the fact that each gp120 trimer must engage not only CD4, but almost immediately thereafter, a CKR as well (typically CCR5)[1,6]. Moreover, once gp120 binds to CD4 and CKR, it quickly dissociates from gp41. If this occurred in a non-concerted manner among the three members of the trimer spike, that spike would be rendered nonfunctional (i.e. noninfectious). Perhaps this loss of gp120 is obviated by an apparent excess of gp120 present in the virion surface tufts[19]. In any case, we will exclude consideration of trimer inactivation by unsuccessful partial dockings – a simplification that works against our main argument.

The probability of a successful trimer docking with three different CD4 molecules and three different CKR molecules at the surface of a potential host cell is, theoretically, exponentially proportional to the surface density and stoichiometry of appropriately oriented CD4 and CKRs. To get some sense of the importance of receptor density, we can make certain additional simplifying assumptions, all of which are biased against our central thesis by underestimating the advantage of clustering. For example, we shall assume that spontaneous, appropriately spaced 1:1 association occurs between CKRs and CD4 in the absence of gp120, although the evidence for this is limited and may not apply to CXCR4[32-34].

This assumption greatly simplifies the problem of successful HIV binding, as it assures coordinated involvement of both coreceptors each time gp120 engages CD4. It also minimizes the theoretical need for CD4 (and, hence dual-receptor) binding, since cross-linking of CKRs alone should result in subsequent capping and polarized increases in receptor density. In reality, CD4 and gp120 are not present in fixed pairs, and binding of gp120 may be required to anchor the virus to the surface via high-affinity interactions, giving it time to form and stabilize much weaker interactions between gp120 and CKRs. Secondly, cross-linking CD4 on resting cells may be sufficient to induce membrane fusion of preformed vesicles containing CKRs, thereby increasing the surface density of these key receptors. Thirdly, initial binding to CD4 may allow the virus to probe receptor density without springing a gp41 molecule. This would minimize the loss of infectivity from unsuccessful gp120 – CD4 interactions on unsuitable host membranes. Fourthly, the greatly decreased probability of docking with six as opposed to three receptors may serve as an important additional barrier to fusion, in contrast to the model we are discussing with its simplifying assumption of pre-associated coreceptor pairs on resting cell membranes.

The average size of an HIV particle is ~120 nM in diameter[19]. Assuming an optimal 1:1 ratio of CD4 and CKR surface molecules per activated cell at ~30,000/cell for each, and an activated T-cell surface area of ~300 uM^2, random distribution of receptors (or 1/10,000 nM^2) will not result in the necessary receptor aggregates occurring with sufficient frequency in the semi-fluid external plasma membrane for any given virion to have much chance of encountering the necessary three pairs of coreceptors in an appropriate configuration. It should also be noted that the calculated surface area for the lymphocyte is based on a smooth surface, which grossly underestimates the surface area on the highly ruffled membrane of the activated T-cell.

Thus, even after making several simplifying assumptions favoring the chance of successful virus-host fusion, we are still left with the conclusion that only a dramatic increase in surface density over the resting state can provide adequate numbers of CD4 and CKR to ensure that binding of three viral heads will occur in a sufficiently concerted manner to release the gp41 trimer before the loss of individual non-covalently linked gp120 chains renders the involved envelope spike nonfunctional. In other words, the HIV envelope spike is intrinsically capable of interrogating the putative host-cell surface for density of appropriate receptors. If this surface receptor density increased dramatically with the ability of the cell to support a full cycle of HIV replication, it could serve as the necessary discriminator between internally suitable (i.e. activated and healthy cells) vs. unsuitable (i.e. resting, dying, or fragmented) cells.

Aggregation vs. upregulation

Greatly increased receptor density might be achieved at the cell surface either by expression of much higher levels, or by aggregation and focus of the existing surface receptors at the site of virus binding. There is no large increase in sCD4 upon activation of T-cells. By contrast, CKRs have been shown to increase surface expression rapidly, by several fold upon even minimal stimulation via fusion of vesicles carrying preformed CKRs with the external plasma membrane[4-6,35]. Thus, cell activation may increase one key component of the receptor complex. Nevertheless, our calculations above suggest that distribution of thousands of coreceptor micro-aggregates

evenly over the cell surface would still not yield adequate receptor density for efficient viral binding and entry. Furthermore, as noted above, published experimental data do not support a strict relationship between total cell surface receptor number and virion binding[6]. This is consistent with the ability of cells to focus receptors for HIV within limited surface areas at densities much greater than those calculated for random distribution. We therefore turn our attention to macro-aggregation, or capping, of receptors at the cell surface.

First described for B-cells binding cognate antigen, capping is now recognized as a general mechanism for focusing the interaction between immune cells, allowing highly efficient communication and restricting the release of potentially toxic components to bystander cells. The general term "immunological synapse" has been coined in reference to the physical partitioning of key adhesion and receptor molecules at focused sites of cell-cell interaction. Furthermore it has been shown that HIV exploits this immunological synapse during cell-to-cell transmission[13,15,16]. We and others have demonstrated that HIV envelope applied *in vitro* to CD4+ T-cells is sufficient to induce co-capping of HIV receptors to a leading edge pseudopod prior to chemotaxis[14,18]. While it is doubtful (albeit, not inconceivable) that an individual virion could trigger receptor co-capping by engagement of only one or two surface receptors, it is not difficult to imagine the release of gp120 from a focus of infection attracting new host cells. Newly released virions from a single infected cell, or focus of cells, would encounter the leading edge of these chemo-attracted cells, and have the opportunity to bind capped regions of high CKR and CD4 density.

Actin as a key factor

For this scenario to work in favor of HIV as envisioned, it is necessary for resting cells and dying cells (or cell fragments) to be incapable of co-capping receptors and/or responding to a chemotactic gradient. First, by definition, resting cells do not exhibit chemotaxis, although they may, in some cases, be triggered by chemotactic signals to activation and response[36]. Second, and crucial to this discussion, cells also lose the ability to polymerize actin at the inner cell membrane in response to external receptor engagement early in the process of apoptosis[37-40]. In some cases this is because actin polymerization has been recruited and redirected to specific sites of external membrane blebbing, or internal organelle disruption[38,39]. Not only does loss of stimulus-responsive actin polymerization eliminate the possibility of chemotaxis and diapedesis, it also prevents capping of surface molecules.

Thus, any activated cells which are present at sites of virion release will not present high-density, co-capped coreceptors if they have already initiated programmed cell death. Similarly, apoptotic blebs could not initiate co-capping of any receptors carried on their surface. One report of synthetic vesicles triggering viral fusion and core release[41] would appear to argue against the need for the proposed mechanisms, but the vesicles artificially generated in that study had very high densities of CD4 and CKR co-receptors, far exceeding the levels expected to be present on apoptotic blebs or cells, thereby obviating the need for co-capping.

With minimal modification, the mechanism proposed for cell free virus is adequate to ensure entry into suitable hosts by virions bound via DC SIGN or FcR fixed anti-gp120 Ab to uninfected dendritic cells[13]. Resting T-cells coming into contact with the DC could be activated by cognate recognition of HLA restricted antigen or gp120 itself[15-17]. Alternatively, previously activated T-cells randomly migrating through lymph nodes and sampling DCs would encounter the fixed array of multiple gp120 trimers presented by virions decorating the surface of the DC, and would be stimulated for coreceptor aggregation. As discussed above, only those CD4+ T-cells capable of co-capping their CD4 and CKRs would be able to trigger release of gp41 trimers to initiate membrane fusion.

The proposed model does not absolutely ensure virion selection of viable host cells under all conditions; some virions may still enter resting cells by chance, and experimental data support the possibility of some viral propagation even in such instances[2]. Other virions may not complete a life cycle before programmed cell death is initiated. Nevertheless, even a modest advantage in selecting the proper host cell during each cycle of replication will rapidly translate into an overwhelming preponderance of this feature in the virus of circulating swarms. It is likely that attracting and probing the surface of viable, activated host cells played as important a role in the evolution of HIV's use of CKRs as did the protection of cryptic binding sites from neutralizing antibodies.

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