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FORUM comment

Roles for endocytosis in lentiviral replication

Mark Marsh, Annegret Pelchen-Matthews and James A. Hoxie

Endocytosis is essential for the entry of many viruses into cells. The primate lentiviruses [human immunodeficiency virus (HIV) 1 and 2, and the simian immunodeficiency viruses (SIVs)], however, use endocytosis in other aspects of their life cycles. Here, the authors describe the ways in which the endocytic pathway is used by HIV and SIV and discuss the mechanisms through which endocytosis may contribute to the pathogenic properties of these viruses.

Endocytosis is an essential property of cells. It is also used by many viruses to enter cells[1] and for immune responses to viral infection dependent on major histocompatibility class II (MHC-II). The primate lentiviruses seem not to require endocytosis for entry, but use endocytic pathways for other aspects of their replicative cycle. In this article, we consider the various ways in which endocytosis bears on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection. We consider the role of endocytosis in virus entry, how endocytosis can modulate the surface expression of the viral receptors and, finally, the endocytic properties of the viral envelope glycoprotein (Env).

Endocytosis in HIV entry

Like other retroviruses, lentiviruses consist of a core structure containing the viral genome, surrounded by a lipid membrane bearing multiple copies of the envelope glycoprotein Env. Entry into cells requires fusion of the viral membrane with a cell membrane in a reaction that is usually dependent on the interaction of Env with the principal cellular receptor, CD4 (Ref. 2). The fusion reactions for HIVs are pH independent[1] and, in tissue culture, occur at the cell surface without the need for endocytosis[3,4]. Whether endocytosis facilitates entry of these viruses into T cells, monocytes, dendritic cells and other targets *in vivo* is unclear[5], although it is likely that transcytosis provides at least one route for HIV to cross the rectal epithelium[6].

Cell-surface expression of receptor molecules

CD4 cell-surface expression can be regulated by endocytosis and potentially alter the susceptibility of a cell to HIV infection. The mechanisms controlling CD4 internalization are now understood in some detail[7]. CD4 can undergo rapid and efficient endocytosis through clathrin-coated pits and vesicles. This activity is regulated by phosphorylation-dependent endocytosis signals in the cytoplasmic domain of CD4 and, in lymphoid cells, through interaction with $p56^{LCK}$ – a Src-family protein tyrosine kinase. Association with $p56^{LCK}$ prevents CD4 entry into endocytic vesicles. Phosphorylation of specific serine residues in the cytoplasmic domain of CD4 triggers dissociation from $p56^{LCK}$, thereby removing the restraint to endocytosis. The phosphorylation of CD4 also activates the endocytosis signal that first allows the protein to cluster into clathrin-coated pits, internalize and be delivered to early endosomes. The activated signal also leads to endosomal sorting of internalized CD4 and subsequent delivery of CD4 to late endosomes and/or lysosomes where it is degraded[7].

In HIV- and SIV-infected cells, CD4 cell-surface expression is downmodulated. In the later stages of the infection cycle, downmodulation occurs through the intracellular interaction of CD4 with Env (Ref. 8). Early in the cycle, however, downmodulation is linked to expression of the viral Nef protein[8]. Nef is a small (~27 kDa) virally encoded protein that has been shown to interact with a number of cellular proteins including Src-family kinases (e.g. $p56^{LCK}$), serine kinases[8] and a number of other cell proteins including the β subunit of coatomer[9]. The precise cellular function(s) of Nef remains to be established, but its effects on CD4 surface expression appear to be due to its ability to influence the endocytic properties of CD4 molecules[8]. Nef-induced CD4 downmodulation is independent of serine phosphorylation, suggesting that Nef acts through mechanisms different from those that normally regulate CD4 internalization[8]. Nef may also induce the endocytosis of MHC-I (Ref. 10) and thus may influence the extent to which infected cells are recognized by cytotoxic T cells. Whether Nef downmodulates CD4 and MHC-I expression through similar mechanisms is unclear.

While CD4 is necessary in most cases for HIV and SIV infection, additional cellular molecules are required for virus entry[2]. Recently, several members of the family of seven-transmembrane-domain (7TM), G-protein-coupled receptors for inflammatory chemokines were identified as co-receptors for various HIV-1 strains[11,12]. Significantly, the chemokine ligands for some of these receptors (RANTES, MIP-1α and MIP-1β) have been implicated in protecting individuals from HIV-1 infection[13] and preventing some infected individuals from developing AIDS (Ref. 14). These chemokines appear to prevent viral entry, but their mode of action is unknown. They could directly block interaction of Env with the co-receptor or they could downmodulate the co-receptors from the cell surface. Endocytic modulation has been demonstrated for several related 7TM proteins[15,16], and it is likely that endocytosis will also regulate the surface expression of the chemokine receptors and thereby influence viral infection.

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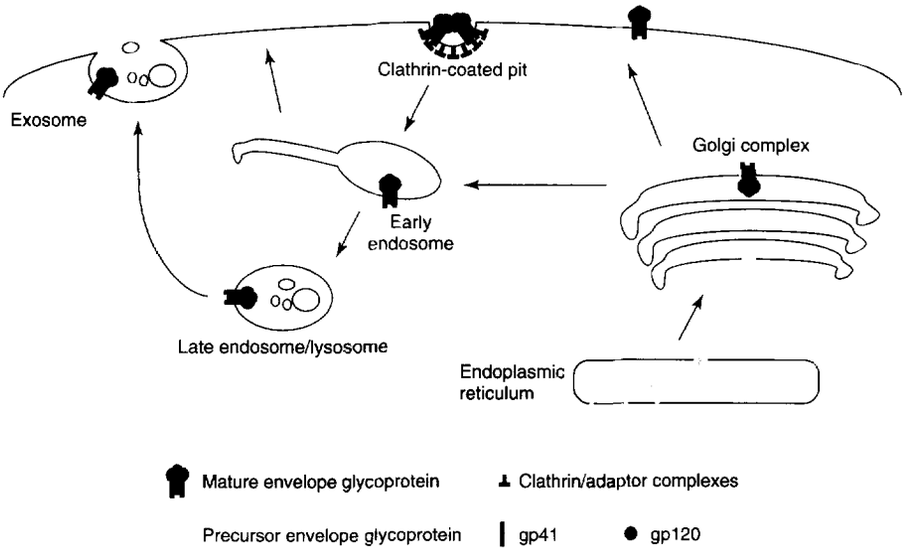

FIGURE 1

Membrane-traffic pathways used by the envelope glycoprotein (Env) of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Env is a type-I integral membrane glycoprotein that is synthesized in precursor form (gp160) on the rough endoplasmic reticulum. The ectodomain comprises the N-terminal ~700 amino acids and is followed by a single, membrane-spanning domain and a C-terminal cytoplasmic domain of ~150 amino acids. The precursor forms oligomers and is glycosylated extensively prior to transport to the Golgi complex and plasma membrane. *En route* to the cell surface, the ectodomain of the protein is cleaved proteolytically to generate the gp120(SU)–gp41(TM) that makes up the mature Env that is expressed on the surface of infected cells and incorporated into virions. Cell-surface Env is internalized constitutively through clathrin-coated pits and delivered to endosomes from where it may either recycle to the cell surface or be diverted to late endosomes and lysosomes. In HIV-infected cells, virions may bud either at the cell surface or into Env-containing late endosomes and lysosomes (MIICs). In the latter case, virions may be released from the cell by exocytosis[39].

Envelope protein expression

Endocytosis is also used by HIV and SIV to regulate the cell-surface expression of Env. Env is the major virally encoded protein of the virion membrane and is responsible for both the receptor-binding and fusion properties of the virus. The pathway of Env synthesis is similar to that described for many viral and cellular membrane proteins (Fig. 1), but lentiviral Envs may have characteristics relevant to the pathology of these particular viruses. For example, much of the HIV-1 Env exported from the rough ER does not accumulate at the plasma membrane or incorporate into virions. Instead, the protein undergoes acid-dependent proteolysis presumably in late endosomes and/or lysosomes[17]. The mechanisms involved in the transport of Env into the endocytic pathway and the significance of these events in the biology of the viruses are unclear.

Many cellular membrane proteins contain tyrosine or dileucine-based signals that mediate endocytosis and other sorting events[18]. The cytoplasmic domains of HIV and SIV Env contain several such sequences that could potentially act as sorting signals, and studies in polarized cells indicate that Env does contain sorting information[19]. SIVs cultured in certain human T-cell lines frequently acquire a mutation that deletes ~130 amino acids from the C-terminus of Env, leaving a predicted cytoplasmic domain of ~20 amino acids. These short-tailed viruses have revealed the location of one such sorting signal. Cells infected with a variant of SIV (CP-MAC, derived from the SIV BK28 molecular clone[20]) express up to 25-fold more Env on their surfaces than cells infected with BK28 (Fig. 2). The increased expression is due to a Tyr-to-Cys change at position 723 in the cytoplasmic domain of CP-MAC Env (Ref. 21). No differences have been detected in the synthesis and transport of the CP-MAC and BK28 Envs, suggesting that events subsequent to delivery to the plasma membrane account for the different surface expression levels. Significantly, the amino acids around Tyr723 have features in common with the Tyr-containing signals identified in other proteins. Moreover, the change is reminiscent of the 'JD' mutation in the low-density lipoprotein receptor (LDL-R), where a similar Tyr-to-Cys change disrupts the endocytosis signal, causing the receptor to accumulate on the plasma membrane[22].

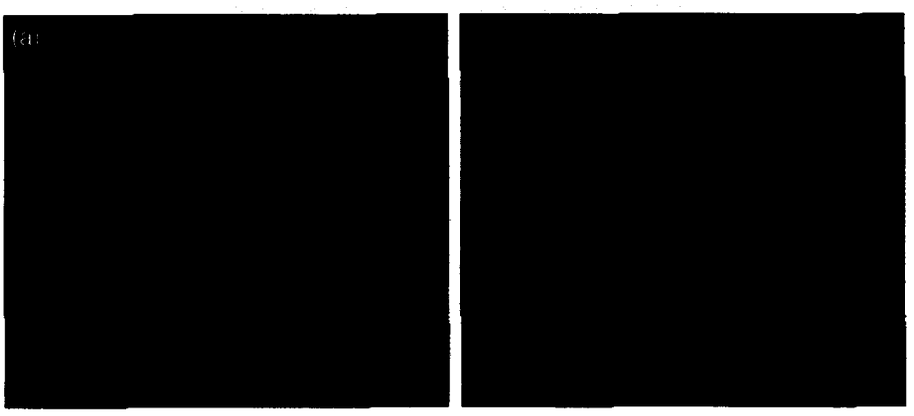

FIGURE 2

Human T cells infected with simian immunodeficiency virus (SIV) BK28 or CP-MAC viruses. T cells were infected with SIV BK28-derived viruses (a) encoding an envelope protein with Tyr at position 723, or CP-MAC (b) encoding an envelope protein with Cys at position 723. The infected cells were fixed, permeabilized and stained with an antibody against the envelope protein. The CP-MAC envelope protein is located on the cell surface, whereas the BK28 envelope is primarily intracellular.

The BK28 Env, expressed in the absence of other SIV proteins, is actively internalized from the cell surface. By contrast, the CP-MAC Env, containing the Tyr723Cys mutation, is internalized less efficiently[23]. When the cytoplasmic domain of SIV Env is used to replace the cytoplasmic domain of CD4, the Env signal is as efficient as the LDL-R endocytosis signal in mediating endocytosis[23]. Furthermore, electron microscopy shows that a Tyr723-containing Env associates with coated pits and is likely, therefore, to use the clathrin-dependent endocytic pathway.

Do similar signals exist in full-length Envs that are more representative of viruses *in vivo*? On cells infected with SIVmac239, the cell-surface expression of the full-length Env is also increased

when the analogous Tyr (position 721) is mutated[21], although the increase is less (4–5 fold) than that seen for viruses with short cytoplasmic domains. A CD4 chimera containing the full-length SIV tail is internalized efficiently, but the endocytosis of this molecule is only slightly reduced by mutations at Tyr721, suggesting that the full-length tail contains more than one endocytosis signal (A. Pelchen-Matthews, J. Hoxie and M. Marsh, unpublished). Together, these findings indicate that Env carries a functional endocytosis signal.

An analogous tyrosine is conserved in virtually all the primate lentivirus Env sequences (e.g. Tyr712 in many strains of HIV-1), and amino acids surrounding this residue also show a high degree of conservation. Thus, reports that the HIV-1 Env also undergoes internalization[24] suggest that this endocytosis activity is an important and conserved feature of primate lentiviruses. Indeed, similar signals may be present in other retroviral envelope proteins[25].

Why endocytose Env?

Lentiviruses are believed to bud from the surface of infected cells[26] and might be expected to direct Env to this site. Thus, the presence of endocytosis signals that actively clear Env from the plasma membrane appears counterintuitive. In fact, the envelope glycoproteins of viruses such as influenza or respiratory syncytia virus are endocytosed poorly and are expressed in abundance on the surface of infected cells. These latter viruses, however, generally cause acute and self-limited infections, whereas the lentiviruses establish chronic and persistent infections. Env is highly immunogenic and renders infected cells targets for humoral immune responses. For HIV-1 at least, virus production remains high during the course of the infection despite ongoing immune responses[27,28]. Thus, as with other persistent viruses, HIV must have evolved mechanisms that permit virus production in the face of this immune attack.

Although mutations in the ectodomain of Env and in other viral proteins may help HIV escape the immune response, it is possible that virus production in an immunologically competent host depends on the regulated expression of Env on the plasma membrane. As such, determinants that reduce the surface exposure of Env could be advantageous for infected cells and, consequently, virus production. Env internalized from the cell surface could also play a role in triggering MHC-II-dependent cellular immune responses since MHC-II molecules generally acquire their antigens from endocytic compartments[24].

The endocytosis of Env may also play a role in protecting newly infected cells. A consequence of viral fusion at the cell surface is that the Env of entering virions is inserted into the plasma membrane of the target cell. These exogenously acquired proteins could immediately make the cell a target for immunological attack and/or predispose the cell to fuse with adjacent CD4+ cells. Consequently, a signal that would quickly remove these viral proteins might increase the likelihood that an infected cell will survive to produce new virions.

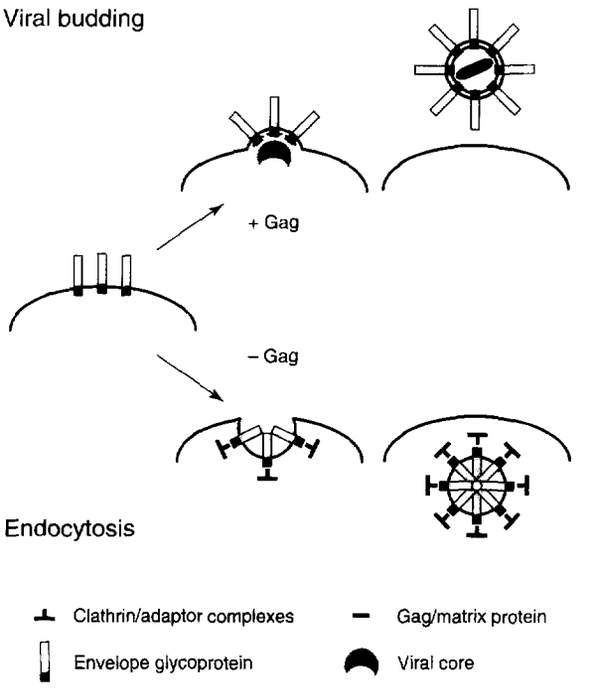

FIGURE 3

Interaction of the envelope glycoprotein Env of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) with coated pits or viral core complexes. In the absence of viral cores, Env delivered to the cell surface will interact with clathrin-coated pits and undergo endocytosis. For virus budding to occur at the plasma membrane, the viral core complexes, containing the viral RNA genome, *gag* gene products, reverse transcriptase and other components essential for viral replication, must interact with the cytoplasmic domains of Env to incorporate Env into the membrane of the budding virion. This interaction may displace or prevent clathrin adaptor interaction with Env, thereby preventing endocytosis and allowing Env accumulation on the cell surface to be linked to viral assembly.

A further possibility is that the endocytosis signals in Env may assist in coordinating the assembly of new virions. If the plasma membrane is the principal site for HIV and SIV budding and cell-surface Env is detrimental to the infected cell, it might be advantageous for the surface expression of Env to be linked temporally and spatially to the assembly process. Such linking could be achieved through the interaction of core components derived from the *gag* gene with the Env cytoplasmic domain[29]. This association may prevent internalization of Env by interfering with the binding of clathrin adaptor molecules (Fig. 3). Recently published evidence indicates that the endocytosis of HIV-1 Env is reduced when Env is expressed together with the HIV Gag protein[30]. Thus, competition between viral (Gag) and cellular (adaptors) proteins for binding sites in the Env cytoplasmic domain will ensure that only those Env molecules being incorporated into virions will accumulate at the cell surface.

The plasma membrane is not, however, the only site of HIV assembly. There are reports that HIVs and SIV can bud into intracellular vesicles (see for example Refs 31–34). By analogy to other viruses, such as bunya- and corona-virus, these vesicles are often considered to be derived from exocytic compartments[26]. A recent study has indicated that, in HIV-1-infected

monocytes, viruses bud primarily into intracellular vesicles (M. Moore, pers. commun.). Moreover, immunoelectron microscopy has demonstrated that these vesicles are MHC-II-enriched compartments (MIICs) – the late endosomes and lysosomes implicated in loading MHC-II with peptides[35]. How can virus bud into endocytic organelles? Experiments in polarized cells indicate that Env directly influences the site of budding. When Gag is expressed alone in such cells, Gag particles are released into the apical and basal medium, but, when Gag and Env are expressed together, particles only appear in the basal medium[36,37]. Significantly, the amino acids around Tyr723 in BK28, and the corresponding residues in HIV, resemble several well-characterized lysosome-targeting signals[18,38]. The signal in Env may function, therefore, in both endocytosis and targeting to late endosomes/lysosomes (Fig. 1) and, in some cell types at least, may facilitate budding at this location. The infectious potential of virions assembled on intracellular membranes and their relative contribution to the total population of infectious viruses is unknown. However, the recent observation that MIICs can fuse directly with the plasma membrane[39] raises the possibility that the release of intracellular virions might be regulated. Furthermore, the assembly and release of virus from MIICs may be related to the observation that MHC-II can be found in abundance in HIV membranes[40].

If endocytosis signals in Env are as important as we propose, it might be anticipated that mutations that disrupt these signals would have an impact on virus infection and pathogenesis. Experiments in tissue-culture systems indicate that SIV mutants containing the Tyr723Cys mutation show a marked increase in their infection kinetics and cytopathic effect[21,23]. Importantly, in the SIV system, the consequences of mutations that disrupt the endocytosis signals can be evaluated in animal models. It will be of interest to determine whether viruses with selective mutations in these signals are altered in their virulence, immunogenicity and/or ability to establish persistent infection.

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