Labeling HIV-1 virions with two fluorescent proteins allows identification of virions that have productively entered the target cell

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Abstract

GFP–Vpr labeled HIV-1 virions have provided a method to visually examine the interactions between the virus and target cell during infection. However, existing methods to discriminate between virions that have been non-specifically endocytosed from those that have productively entered the host cell cytoplasm have remained problematic. Therefore, we examined the ability of a series of membrane-targeted fluorescent fusion protein constructs to be incorporated into virions. We find that a fluorescent protein fusion targeted to the plasma membrane by the addition of the N-terminal 15 amino acid sequence of c-Src (S15) is efficiently packaged into HIV virions. Using fluorescent proteins fused to this sequence, we have generated virions dually labeled with S15–mCherry and GFP–Vpr. Importantly, we can detect the loss of this S15–mCherry membrane signal following fusion.

After infection with VSV-g pseudotyped HIV virions, we find a measurable, specific loss of membrane label during infection. This loss of fluorescence is not observed when fusion is prevented using bafilomycin A. This increased ability to discriminate between non-productively endocytosed virions and those actively undergoing steps in the infectious process will facilitate efforts to examine early steps in infection microscopically.

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Introduction

Infection of a host cell by HIV-1 requires fusion of the viral membrane with the membrane of the host cell, thereby releasing the viral core into the host cell cytoplasm. Once in the cytoplasm, the viral core undergoes a series of trafficking and replicative events required to establish infection. To access nuclear host cell DNA, the HIV-1 core must be directionally trafficked towards the nucleus. During this time, the virus undergoes the poorly understood process of uncoating, which is the sequential loss of viral proteins from the preintegration complex (PIC) containing the viral genome. During this time, the viral RNA genome is converted to DNA, and this DNA is ultimately imported into the nucleus of the target cell and integrated into the host cell chromosome (reviewed in Dvorin and Malim, 2003). However, studies examining these events have lagged behind other areas of HIV research such as viral fusion, gene regulation and virus production because the latter have been more easily studied using classical biochemical and molecular biology approaches. In recent years fluorescent microscopy has become an invaluable tool to examine the intracellular trafficking and early replicative events of viruses during infection (reviewed in Campbell and Hope, 2005; Dohner et al., 2005). However, it has been shown that, in the case of HIV-1, nonspecific receptor-independent uptake of HIV virions occurs at high frequency (Marechal et al., 1998; McDonald et al., 2002; Schaeffer et al., 2001). Therefore, analysis of the intracellular trafficking of enveloped virions requires the ability to discriminate between virions that have fused with the host cell membrane from virions that have nonproductively entered the host cell via endocytosis.

Our lab has previously examined the intracellular behavior of HIV by fluorescently labeling the HIV core with a GFP–Vpr...
fusion protein and labeling the virion membrane with the fluorescent, membrane intercalating dye DiD (McDonald et al., 2002). However, the efficiency of DiD labeling of virus can vary considerably between different viral preparations. We therefore sought to develop another method to label the membrane of virions with increased specificity and consistency. To this end, we examined the incorporation of a series of GFP fusion proteins targeted to the plasma membrane or otherwise known to be incorporated into HIV virions. We find a fluorescent fusion protein containing the 15 N-terminal amino acids of Src (Rodgers, 2002) is efficiently incorporated into HIV virions and is lost following fusion of the viral membrane with the host cell membrane, thus providing a powerful tool to identify fluorescently labeled HIV virions that have productively entered the host cell.

**Results**

**Incorporation of fluorescent fusion proteins into HIV virions**

We first wanted to examine the ability of specific GFP-fusion proteins to be incorporated into HIV virions. A series of fusion proteins were chosen for their ability to associate with the host cell plasma membrane or for their ability to otherwise be incorporated into HIV virions. These fusion proteins were then coexpressed in virus-producing 293T cells also expressing an mRFP–Vpr fusion protein to label HIV-1 cores. Specifically, the membrane associated proteins examined were GFP derivatives containing the farnesylation sequence of p21(Ras) (EGFP-F) (Harvey et al., 2001), another containing the 10 N-terminal, membrane targeting amino acids of the Src tyrosine kinase p56<sup>lck</sup> fused to GFP (L10–GFP) (Rodgers, 2002; Rodgers et al., 1994; Shenoy-Scaria et al., 1993), and one containing the 15 N-terminal membrane targeting amino acids of p60<sup>co-Src</sup> fused to GFP (S15–GFP) (Rodgers, 2002; Shenoy-Scaria et al., 1993). GFP fusions to the viral accessory protein Nef (Nef–GFP) and the cellular protein Cyclophilin A (CypA–GFP) were also examined since each has been reported to be incorporated into HIV virions. These fusion proteins were then analyzed for incorporation into HIV virions, as well as for their ability to otherwise be associated with the host cell plasma membrane or for their ability to otherwise be incorporated into HIV virions. These fusion proteins were then coexpressed in virus-producing 293T cells also expressing an mRFP–Vpr fusion protein to label HIV-1 cores. Specifically, the membrane associated proteins examined were GFP derivatives containing the farnesylation sequence of p21(Ras) (EGFP-F) (Harvey et al., 2001), another containing the 10 N-terminal, membrane targeting amino acids of the Src tyrosine kinase p56<sup>lck</sup> fused to GFP (L10–GFP) (Rodgers, 2002; Rodgers et al., 1994; Shenoy-Scaria et al., 1993), and one containing the 15 N-terminal membrane targeting amino acids of p60<sup>co-Src</sup> fused to GFP (S15–GFP) (Rodgers, 2002; Shenoy-Scaria et al., 1993). GFP fusions to the viral accessory protein Nef (Nef–GFP) and the cellular protein Cyclophilin A (CypA–GFP) were also examined since each has been reported to be specifically incorporated into HIV virions (Bukovsky et al., 1997; Franke et al., 1994; Thali et al., 1994; Welker et al., 1998). A GFP fusion targeted to the outer membrane of cells via a glycosyl-phosphatidylinositol (GPI) anchor was also examined but strongly inhibited virus production, and was therefore not studied further (data not shown). Unfused GFP was included as a control to determine the specificity of the virion incorporated fluorescence observed in these experiments. Virus collected from cells expressing the above GFP fusions was bound to glass coverslips, stained for p24 and imaged. mRFP–Vpr signal was observed to overlap with p24 staining, revealing that mRFP–Vpr is incorporated into virions (Fig. 1B). When the GFP fluorescent intensity of mRFP–Vpr+ p24+ HIV-1 virions was examined, minimal incorporation of L10–GFP and CypA–GFP was detected. However, measurable incorporation of Nef–GFP, EGFP-F and S15–GFP into HIV virions was observed. Of these three proteins, S15–GFP incorporation was highest in terms of average virion fluorescence (Fig. 1A) and the percentage of virions labeled (Fig. 1B). Similar results were obtained when the S15 leader sequence was attached to the recently developed red fluorescent protein mCherry (Shaner et al., 2004) and virion cores were labeled with the better characterized GFP–Vpr protein (McDonald et al., 2002) (Fig. 1C). Virion labeling with either S15–GFP or S15–mCherry typically resulted in detectable fluorescence in greater than 90% of virions as determined by p24 staining. By contrast, labeling with GFP–Vpr or mRFP–Vpr typically generated detectable fluorescence in 40–80% of virions as determined by p24 staining under these conditions (data not shown). In the absence of HIV transfection, supernatants from S15–mCherry transfected 293T cells did not release appreciable amounts of labeled membrane into the supernatant when these supernatants were exposed to glass coverslips as in Figs. 1B, C (data not shown). S15 expression in the virus-producing 293T cells did not negatively affect virus production (data not shown). Moreover, when equivalent amounts of a GFP reporter virus were used to infect HeLa cells, virus preparations labeled with GFP–Vpr, S15–mCherry or both proteins remained as infectious as unlabeled VSV-g pseudotyped virus (Fig. 1D).

**S15–mCherry localizes to areas of microvilli and cell projection**

We next sought to examine the intracellular localization of S15–mCherry in transfected cells. S15–mCherry expressing 293T cells were fixed and stained for ezrin. Ezrin is a member of the ezrin radixin moesin family of protein that links the actin cytoskeleton to the plasma membrane (Bretscher et al., 2002). Our lab has found that ezrin is present on microvilli and other cell protrusions where CD4 and CCR5 accumulate (Steifens and Hope, 2003) as do constructs expressing HIV matrix and matrix–capsid fused to GFP (Gomez and Hope, 2006). As expected, S15–mCherry was observed to localize primarily to the plasma membrane, accumulating in ezrin containing structures in 293T cells (Fig. 2), while a small amount localized to discreet intracellular vesicular compartments.

**Loss of S15–mCherry from HIV virions correlates with fusion with target cells**

Since S15–mCherry was able to label virion membranes with high efficiency (Fig. 1), we next sought to determine if the S15–mCherry signal was lost from GFP–Vpr labeled virions upon fusion with target cells. We therefore infected HeLa cells with VSV-g pseudotyped HIV virions dually labeled with S15–mCherry and GFP–Vpr. We chose VSV-g envelope because it fuses with target cells at high efficiency and can be effectively inhibited by drugs such as bafilomycin A1 (BafA) that inhibit endosome acidification (Bowman et al., 1988; Yoshimori et al., 1991). To synchronize entry of virus into target cells, HeLa cells were spinoculated at 17 °C to allow virion binding but prevent virion endocytosis (O’Doherty et al., 2000). Following spinoculation, cells were transferred to 37 °C in the presence or absence of BafA. These cells were then fixed at various times post-spinoculation and imaged. In cells pretreated with bafilomycin A (BafA), which prevents fusion of VSV-g pseudotyped
virions, most GFP–Vpr+ virions were still labeled with S15–mCherry 2 h post infection (PI) (Fig. 3A). In the absence of drug, however, while some S15–mCherry labeled virions were still clearly visible 2 h post infection, a population of GFP–Vpr+ virions that lacked S15–mCherry labeling had emerged (Fig. 3B). We conclude that productive entry into target cells results in the loss of S15–mCherry signal from HIV-1 virions.

**Kinetic analysis of HIV-1 infection of target cells using S15–mCherry**

The ability to discriminate between virions that have productively entered the target cell from those that have not allowed us to examine the fate of both of these viral populations over time. When infection was mediated by spinoculation at 17 °C, cells fixed immediately after spinoculation maintained their S15–mCherry signal in the presence or absence of BafA (Fig. 4A). The viral supernatant was removed from the remaining coverslips, replaced with fresh media and temperature was shifted to 37 °C. When these cells were fixed 1–4 h PI, the overall number of GFP–Vpr+ virions was observed to decrease over time. In contrast there was no decrease in the number of GFP–Vpr labeled particles after 4 h in the presence of BafA (Fig. 4A). BafA treatment prevented both the reduction of virion number and loss of S15–mCherry labeling (Fig. 4A), suggesting that endosomal acidification is required both for fusion of VSV-g pseudotyped virions and degradation of virions which do not achieve productive entry to the target cell. After 1 h of incubation at 37 °C the total number of GFP–Vpr positive particles decreased by approximately 50%. Analysis of associated S15–mCherry signal at the 1 h time point revealed a decrease in the number of S15–mCherry+ virions. This was mirrored by an increase in the number of virions lacking S15–mCherry signal representing viral cores that had entered the cytoplasm (Figs. 4A, B). Following this increase in the number of cytoplasmic virions observed at 1 h PI, this viral population remained largely intact 2 h PI and was then observed to decline by 4 h PI (Figs. 4A, B). The unfused, S15–mCherry+ population, however, was observed to steadily decline between 1 and 4 h PI, so that by 4 h, the vast majority of cellular virions had lost their S15–mCherry membrane label (Figs. 4A, B). The analysis of the ratio of the S15–mCherry labeled GFP–Vpr signal to those that had lost their membrane label in the remaining viral population is shown in the insert in Fig. 4B.

This analysis reveals that initially, less than 5% of the virions were S15–mCherry negative. In contrast after 4 h the S15–mCherry negative virions represented more than 80% of the remaining GFP–Vpr labeled population. Similarly, when HIV-1 virions lacking envelope were used to infect target cells, the S15–mCherry signal was not lost. However, in this case, the number of virions was observed to decrease over time (data not shown). We conclude that the loss of S15–mCherry signal from GFP–Vpr labeled virions correlates with the ability of virions to fuse with target cells and therefore identifies virions that have productively entered the target cell.

**Discussion**

In this study, we find that the attachment of the 15 N-terminal amino acids of c-Src can drive incorporation of fluorescent fusion proteins into budding HIV virions. Targeting GFP to the plasma membrane by other sequences had limited ability to
facilitate virion incorporation (Fig. 1), suggesting that the specific membrane regions targeted by S15 fusion constructs is necessary for virion incorporation. This stretch of amino acids contains a myristoylated Gly2 residue followed by a string of basic amino acids known to be responsible for the membrane association of c-Src (reviewed in Resh, 1994). It has been shown that association of Nef with the plasma membrane and subsequent incorporation into HIV virions depends on a similar stretch of basic residues flanking a myristoylated glycine (Welker et al., 1998). It was therefore not surprising that both of

Fig. 3. Virion incorporated S15–mCherry signal is lost following productive entry into target cells. HeLa cells were spinoculated (O’Doherty et al., 2000) with VSV-g pseudotyped, S15–mCherry and GFP–Vpr labeled R7ΔEnv virions for 2 h at 17 °C. Virus was removed and replaced with 37 °C media. Cells were incubated at 37 °C, fixed at the indicated time PI and imaged. GFP positive puncta were than quantified and individually examined for the presence of above background mCherry signal. (A) Representative experiment in which the number of virions observed per 10 Z-stack fields was calculated at the indicated time points in the presence or absence of BafA. Grey bars indicate the number of S15–mCherry positive virions and black bars indicate the number of S15–mCherry negative virions. Numbers above each time point indicate the percentage of GFP–Vpr positive virions lacking S15–mCherry signal. (B) Total number of virions compiled from pooled data derived from 3 independent experiments. The input virion level was normalized to 100% in order to accommodate differences in initial observed viral input. The changes in the populations of total virions (♦) S15–mCherry positive virions (●) and S15–mCherry negative (▴) virions over time is shown. (Panel B inset) The relative amount of S15–mCherry positive virions (●) and S15–mCherry negative (▴) virions as a percentage of the remaining viral population. Error bars represent the standard error of the mean of three experiments.

Fig. 4. Temporal analysis of S15–mCherry loss following infection. HeLa cells were spinoculated with VSV-g pseudotyped, S15–mCherry GFP–Vpr labeled R7ΔEnv virions for 2 h at 17 °C. Virus was removed and replaced with 37 °C media. Cells were incubated at 37 °C, fixed at the indicated time PI and imaged. GFP positive puncta were than quantified and individually examined for the presence of above background mCherry signal. (A) Representative experiment in which the number of virions observed per 10 Z-stack fields was calculated at the indicated time points in the presence or absence of BafA. Grey bars indicate the number of S15–mCherry positive virions and black bars indicate the number of S15–mCherry negative virions. Numbers above each time point indicate the percentage of GFP–Vpr positive virions lacking S15–mCherry signal. (B) Total number of virions compiled from pooled data derived from 3 independent experiments. The input virion level was normalized to 100% in order to accommodate differences in initial observed viral input. The changes in the populations of total virions (♦) S15–mCherry positive virions (●) and S15–mCherry negative (▴) virions over time is shown. (Panel B inset) The relative amount of S15–mCherry positive virions (●) and S15–mCherry negative (▴) virions as a percentage of the remaining viral population. Error bars represent the standard error of the mean of three experiments.
these fluorescent fusions were specifically incorporated into HIV virions to similar degrees (Fig. 1A). However, it has been reported that the S15–GFP protein is associated exclusively with the detergent soluble membrane (DSM) fraction (Rodgers, 2002). Alternatively, it has been reported that the HIV structural precursor protein Pr55Gal localizes initially to the DS fraction but subsequently localizes to the detergent-resistant membrane (DRM) fraction containing glycolipid enriched membrane, or lipid rafts, prior to virion release (Ono and Freed, 2001). Despite this minor discrepancy, our microscopic analysis indicates that S15–mCherry localizes to ezrin containing membrane structures (Fig. 2), in which Gag–GFP is also known to accumulate during assembly (Gomez and Hope, 2006), demonstrating that the S15 sequence directs proteins to the area of the plasma membrane that supports viral assembly.

To date, the preferred method to induce incorporation of non-viral proteins into HIV virions has been the generation of fusion proteins tethered to the viral Vpr protein (Cavrois et al., 2002; McDonald et al., 2002; Sato et al., 1995; Wu et al., 1995). The attachment of the S15 leader sequence onto non-viral proteins similarly provides an alternative mechanism to drive virion incorporation. In fact, this method may prove to be more effective than Vpr fusions. In these experiments, membrane labeling directed by the S15 sequence always labeled a higher percentage of virions than was observed by the Vpr fusion protein, regardless of the combination of fluorescent proteins used (data not shown). This is likely because high levels of Vpr expression in virus-producing cells can negatively affect virus production (data not shown). Therefore, inducing virion incorporation of non-virion proteins by generation of fusion proteins containing the S15 sequence may prove more effective than Vpr in some cases, especially when there is no need for the protein to remain associated with the HIV preintegration complex following fusion. Such a mechanism for driving virion incorporation of non-virion proteins by inducing their membrane association may likely be achievable using other membrane targeting sequences and is likely not specific to HIV. Others have observed that GFP incorporation into virions can be achieved by tethering GFP to the membrane targeting sequence of HIV-1 Nef (Welker et al., 1998), consistent with the results observed in Fig. 1A. Moreover, a palmitoylated form of yellow fluorescent protein (YFP) has been shown to be efficiently incorporated into murine leukemia virus (Melikyan et al., 2005). In this regard, fluorescent proteins targeted to different sites in the membrane may therefore provide insight into the specific membrane microdomains used as assembly platforms by different viruses.

The ability of dually labeled virions to serve as a marker for viral fusion represents a significant step in our ability to track the interactions of the viral preintegration complex with the host cell. S15–mCherry signal is retained when virion fusion is prevented with BafA, which inhibits fusion mediated by pH dependent envelopes such as VSV-g, and following endocytosis of HIV-1 virions lacking an envelope protein, demonstrating that loss of S15 associated membrane signal occurs only when fusion occurs. Using this system to monitor fused and unfused viral populations over time following synchronized infection, we observed the disappearance of detectable cellular virions. Most of this loss occurs rapidly and represents the loss of the S15–mCherry positive viral population in the first hour post infection. This loss cannot completely be accounted for by conversion of this population to a cytoplasmic S15–mCherry negative population, but can be prevented with BafA. This suggests that many virions are rapidly degraded following endocytosis before they can enter the target cell, even when virions are pseudotyped with VSV-g.

Further work is required to determine the fate of GFP–Vpr labeled virions following fusion. By 4 h PI, we observed a significant reduction in the number of cytoplasmic virions compared to earlier time points. Three factors could be contributing to the loss of cytoplasmic GFP–Vpr labeled cores that we observed over time in these experiments. Most obviously, the loss of GFP–Vpr signal could represent the fact that the viral DNA has been imported into the nucleus. This would include the loss of GFP–Vpr association from the PIC, as we do not observe GFP–Vpr positive punctate in the nucleus of target cells (data not shown). This is consistent with other work, which has measured nuclear viral DNA as early as 3 h following an unsynchronized infection (Bukrinsky et al., 1992). Second, there may be a proteasome-dependent mechanism by which cytoplasmic virions are degraded. This is consistent with previous reports showing an antiviral activity of the proteasome in HIV-1 infection (Schwartz et al., 1998), as well as our own data showing that proteasome inhibition does increase the stability of GFP–Vpr labeled virions over time (data not shown). Third, GFP–Vpr association with the viral PIC may be lost or become reduced during the process of uncoating and cDNA generation. Lastly, the loss of fluorescent signal we measured may be a result of pH quenching of fluorescent proteins within the lysosomal compartment. Such quenching has been reported previously, although it was also found that this quenching was rapidly reversible upon return to neutral pH (Melikyan et al., 2005). This effect would result in an underestimation of the number of unfused virions, although these virions would be subjected to conditions that would rapidly lead to their degradation in the absence of VSV-g mediated fusion. More work is required to understand how much each of these components contributes to the loss of GFP–Vpr labeled virions we observed in these experiments. The ability to discriminate between virions that have fused with the target cell, combined with the ability to synchronize infection by spinoculation, will allow these issues to be examined in more detail. Moreover, these methods will also facilitate the direct observation of early events during infection, as well as allow analysis of the protein composition of the viral preintegration complex, which should lead to an in situ characterization of the still poorly understood process of uncoating.

Materials and methods

Cells and pharmaceuticals

293T and HeLa cells (American Type Culture Collection) were cultured at 37 °C, 7% CO2 in Dulbecco’s modified
Eagle’s medium (Hyclone) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine and 10 μg/ml ciprofloxacin. Bafilomycin A1 (Sigma) was prepared in DMSO and diluted to a final concentration of 20 nm in DMEM.

Virus and infections

To screen virion incorporation of fusion proteins, 293T cells in 6-well plates were transfected with 1.75 μg of the indicated fusion protein expression plasmid was cotransfected with 1.75 μg R7Δenv GFP, 1 μg VSV-g and 0.1 μg mRFP–Vpr using polyethylenimine (PEI) (MW 25,000, Polysciences). To generate virus uses in infection, 10-cm plates of 293T cells were transfected with 7.4 μg R7Δenv GFP, 4.2 μg VSV-g, 0.5 μg GFP–Vpr, and 6.4 μg S15–mCherry using PEI. Virus was harvested 48 h post-transfection. To synchronize infection, target cells were seeded on fibronectin treated coverslips and spinoculated (O’Doherty et al., 2000) with 0.5 ml of virus at a concentration of 50–300 ng/ml for 2 h at 17 °C in the presence or absence of the indicated drug. Virus was then removed and replaced with 37°C media in the presence or absence of the indicated drug and fixed at the indicated time point post infection. In Fig. 1D, the viral inoculum was replaced with fresh media 14 h Pl and GFP expression was measured 48 h later using a FACS Calibur flow cytometer analyzing 5000 events per infection (Becton Dickinson).

Immunofluorescence and microscopy

Virions or cells on glass coverslips were fixed with 3.7% formaldehyde (Polysciences) in 0.1 M Pipes buffer, pH 6.8. Coverslips were stained with α-p24 mAb AG3.0 (NIH AIDS Research and Reference Reagent Program) or α-ezrin (Cytoskeleton Inc.) in blocking solution (10% normal donkey serum, Jackson ImmunoResearch Laboratories or goat normal donkey serum, Cytoskeleton Inc.) or Coverslips were stained with formaldehyde (Polysciences) in 0.1 M Pipes buffer, pH 6.8. Following deconvolution, the number of GFP positive virions was assessed and each virion was individually inspected for punctate mCherry fluorescent signal. Between 9 and 25 images were analyzed for each data point provided. During analysis, channel intensity was increased above the level of the included figures to detect low level fluorescence.

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