Visualization of transfer of a fluorescently-labeled membrane raft protein to T cells using lentivirus

Research Article

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Summary

Lentivirus vector systems have been developed for the safe delivery of foreign genes to target tissues. However, the use of these systems for delivering specific proteins to target cells has been largely unexplored. To test this concept, the lentivirus expression plasmid pLenti was utilized to overexpress in producer cells a YFP-fusion protein that is specifically targeted to glycolipid-enriched membrane rafts, which is the site of virus assembly. Our data show that virus generated in producer cells that expressed the YFP fusion protein were able to effectively label target cells by a 2-3 hr incubation with the virus. Labeling of the target cells was specific to the lentivirus, as it was blocked by pre-incubating the virus with antibody to the surface protein, and it was not affected by pre-treating the target cells with cyclohexamide. T cells that were labeled using the lentivirus underwent a robust stimulation following crosslinking the T cell receptor, thus showing that T cells labeled using lentivirus remained responsive to extracellular cues. Altogether, these results show that overexpression of foreign proteins in lentivirus producer cells can yield protein-loaded viruses, which can then function to deliver the protein to target cells. Thus, our findings suggest an avenue for targeting specific proteins to cells where foreign gene expression is not feasible.

I. Introduction

HIV-based lentiviral vectors represent an emerging tool in gene therapy, as they offer important advantages over retroviral vectors in gene delivery to target cells (Lever et al, 2004; Wiznerowicz and Trono, 2005). Towards this end, HIV-based vectors with improved biosafety have been developed for generation of lentivirus using transected producer cells (Poznansky et al, 1991; Shimada et al, 1991; Reiser et al, 1996; Naldini et al, 1996; Dull et al, 1998). Production of lentivirus in these systems is dependent upon efficient packaging of the virus by the producer cells. In the case of HIV, packaging of virus is dependent upon the glycolipid-enriched membrane rafts that occur in cell membranes (Nguyen and Rildreth, 2000; Ono and Freed, 2001; Manes et al, 2003). Lipid rafts are a distinct class of membrane domains that are composed of cholesterol, sphingolipids, and specific proteins (Simons and Toomre, 2000; Brown and London, 1998). The precise mechanism by which rafts function in viral production is not known, but it may be related to rafts providing an optimal site for assembly of virus particles and their budding (Manes et al, 2003).

The observed incorporation of rafts and raft-associated proteins in the envelope of lentivirus (Nguyen and Hildreth, 2000; Manes et al, 2003) suggests that the viruses may represent an effective tool for delivering proteins to target cells from producer cells. For example, just as HIV may deliver endogenous proteins from the host cells in order to improve their infectivity (Manes et al, 2003), foreign gene expression in producer cells may lead to loading of the gene product in the viral envelope for its delivery to target cells. Such direct delivery of foreign proteins to target cells by lentivirus could have important therapeutic applications. For example, cells that are deficient in a key enzyme could have the protein delivered directly by the virus, rather than relying on gene expression in the target cell.

To test the notion of delivery of foreign protein to target cells using lentivirus, we utilized a recently developed HIV-based vector system (Dull et al, 1998) to express and load virus with a membrane raft-associated yellow fluorescent protein (YFP) fusion peptide (Rodgers, 2002). This expression system consists of an HIV-based vector (pLenti) that contains a modified hybrid 5' LTR that...
provides a constitutive transcription of downstream sequences (Dull et al., 1998). We show that pLenti generated abundant expression of the YFP protein in producer cells, and produced virus that effectively labeled target cells during a 2-3 hr incubation. Expression of the YFP gene in producer cells trans to pLenti by co-transfection with a separate plasmid also generated virus that labeled target cells. Altogether, our results suggest a novel pathway for directly delivering foreign protein to target cells, and that may facilitate therapeutic approaches in systems that are not adaptable to foreign gene expression.

II. Materials and Methods

A. Generation of lentivirus

All reagents for production of lentivirus were purchased from Invitrogen (ViraPower™ Lentiviral Gateway™ Expression kit, Invitrogen, Carlsbad, CA). L10-YFP in the lentivirus expression plasmid pLenti (L10-YFP/pLenti) was generated by subcloning L10-YFP from pWay20 into an EcoRI site that was inserted into pLenti using LR clonase (Invitrogen) recombination reaction from pENTR4 (Invitrogen). Correct orientation of L10-YFP in pLenti for expression by the RSV/5’ LTR and CMV promoters was screened by transient expression in HeLa cells as described, and confirmed by DNA sequencing.

293FT producer cells (Invitrogen) were maintained in DMEM containing 10% FCS and antibiotics. Lentivirus was produced by co-transfecting 293FT cells with L10-YFP/pLenti and ViraPower™ packaging mix per the manufacturer's instructions, using 9 µg of the packaging mix, 3 µg of L10-YFP/pLenti, and 36 µl of Lipofectamine™ 2000. The DNA-lipofectamine mix was added to a 10 cm dish containing 6x10⁶ 293FT cells in 5 ml of Opti-MEM +10% FCS. Fresh media was added on day 2, and the culture was harvested on day 4. For harvesting, the media was collected and media centrifuged at 3000 rpm (GS-6R, Beckman) for 15 min at 4 °C. After which the supernatant was passed through a 0.45 µm filter (Milllex-HV, Millipore, Billerica, MA). The filtrate was then centrifuged at 13,500 rpm (SW 28, Beckman, Fullerton, CA) for 90 min. 4 °C. Virus stock was prepared by suspending the resulting pellet in approximately 0.5 ml of media, which was then aliquoted and stored at -70 °C.

B. Infection

10⁶ Jurkat cells were washed and suspended in 100 µl of RPMI containing 1% FCS plus antibiotics. The cell suspension was transferred to a 96 well plate, and to this was added the indicated volume of virus stock, RPMI containing 1% FCS, and polybrene (Sigma-Aldrich, St. Louis, MO) to a final volume of 200 µl and polybrene concentration of 5 µg/ml. The sample was then incubated at 37 °C for the indicated time, washed with PBS, and then either seeded onto poly-L-lysine (Sigma-Aldrich) coated cover slips for fluorescence microscopy, or resuspended in a minimal volume of media for flow cytometry. For experiments using cell imaging, the virus stock was briefly sonicated (550 Sonic Disembrator, Fisher Scientific, Pittsburgh, PA) prior to adding to the cells.

C. Fluorescence measurement

Microscopy was performed using a Nikon Eclipse E400 fluorescence microscope equipped with an Olympus DW10 camera, and 40X (NA 0.75) and 100X (NA 1.3) objectives. For imaging YFP fluorescence, the samples were excited with wavelengths 465 to 495 nm, and the fluorescence was detected using wavelengths 505 to 555 nm. Flow cytometry was performed using a Becton Dickinson FACSscan, with excitation of the samples at 488 nm, and collection of wavelengths between 515 and 545 nm for the detection of emission.

D. Cell stimulation

Jurkat cells were washed with RPMI containing 50 mM HEPES (pH 7.4), and suspended in the same at a final concentration of 10⁶ cells/ml. The samples were then pre-incubated at 37 °C for 5 min, after which 3 µg of OKT3 was added. The samples were incubated for an additional minute, then sedimented, wash with chilled PBS, and then suspended in 50 µl of SDS-PAGE sample buffer. The samples were separated by SDS-PAGE, and protein tyrosine phosphorylation was measured by immunoblotting with 4G10 (Cell Signaling Solutions, Charlotteville, VA), and detected using enhanced chemiluminescence (Pierce, Rockford, IL).

III. Results

A. Generation of lentivirus in producer cells expressing L10-YFP

The vector used for expression of L10-YFP and generation of lentivirus is illustrated in Figure 1A. The gene encoding L10-YFP was subcloned into pLenti, where expression is driven by a hybrid 5’ LTR composed of the RSV enhancer/promoter in place of the U3 region of the HIV 5’ LTR. The hybrid LTR provides a constitutive, Tat-independent transcription of downstream sequences (Dull et al., 1998). An internal CMV promoter that is immediately upstream of the L10-YFP also drives constitutive transcription (Dull et al., 1998).

Infectious particles were generated by co-transfecting 293FT cells with L10-YFP/pLenti and three other separate and nonoverlapping expression plasmids required for encapsulation and targeting of the virus (Figure 1B): LP1 and LP2, which contain gag and pol, and rev, respectively, and pLP/VSV G, which encodes the G protein of vesicular stomatitis virus (VSV). pLP1, pLP2, and pLP/VSV G were introduced using the ViraPower™ packaging mix (Materials and Methods). Imaging of transfected 293FT cells showed a robust expression of L10-YFP in the producer cells (Figure 1C).

B. Facile and effective labeling of target cells using L10-YFP/lentivirus

Culture supernatants of transfected 293FT cells were harvested 48 hr following transfection, after which the virus was concentrated by centrifugation. Accordingly, a 10 cm plate of 293FT cells was used to generate approximately 0.5 ml of concentrated virus stock. For labeling, a 100 µl aliquot of virus stock was briefly sonicated, and then added to cell cultures for a 3 hr incubation at 37 °C. A fluorescence image demonstrating labeling of Jurkat T cells by incubation with lentivirus from producer cells that expressed L10-YFP is shown in Figure 2A. In separate experiments, Jurkat cells were incubated with lentivirus from 293FT cells that were transfected with an empty pLenti plasmid that did not contain L10-YFP, in which case no labeling of the target cells was detected (Figure 2B). Thus, labeling of the Jurkat cells was specific to virus derived from producer cells that expressed L10-YFP.
YFP. Furthermore, labeling of the Jurkat cells was independent of protein synthesis in the target cells, since treatment of Jurkat cells with cyclohexamide prior to addition of virus had no significant effect on their labeling by the lentivirus (Figure 2C). To further measure for virus-specific labeling of target cells, the virus stock was incubated with antibody to VSV G prior to adding to Jurkat cells. Figure 2C shows that pre-incubation with antibody effectively blocked labeling of target cells. We conclude from these results that constitutive overexpression of the raft-associated protein L_{10}-YFP in lentivirus producer cells results in a virus-dependent transfer of the protein to target cells.

Note that the L_{10}-YFP in Figure 2A is localized in both the outer membrane (arrowheads), and an intracellular compartment underlying the plasma membrane (arrows). Importantly, L_{10}-GFP that is expressed endogenously in Jurkat cells has an identical distribution (Rodgers, 2002). Previous studies of Lck fusion proteins containing GFP have shown that this intracellular compartment represents recycling endosomes (Ehrlich et al, 2002). Altogether, these results indicate that the L_{10}-YFP that is delivered by lentivirus resides in the same cellular pools as protein that is expressed following transfection of Jurkat cells.

Additional characterizations of labeling by lentivirus are shown in Figure 3. These experiments showed that the labeling of target cells was proportional to the amount of virus that was added to the target cells (Figure 3A), as well as the length of time for the incubation (Figure 3B). Interestingly, Figure 3B shows that detectable labeling of the Jurkat cells occurred following an incubation that was less than 1 hr. These results thus underscore the facile nature of the labeling target cells using lentivirus. The linear increase in labeling with time or volume of virus added also suggests that labeling can be finely tuned by adjusting either one of these parameters.

Figure 1. Visualization of L_{10}-YFP in producer 293FT cells transfected with pLenti containing a sequence encoding L_{10}-YFP. (A) Diagram of the lentivirus expression vector pLenti containing L_{10}-YFP (L_{10}-YFP/pLenti). An RSV/5’LTR hybrid promoter in pLenti drives a constitutive, Tat-independent expression of L_{10}-YFP. Other elements of the vector include an HIV-1 packaging signal (Ψ), Rev response element (RRE), and a 3’ LTR containing a deletion of its U3. The modified 3’ LTR provides a self-inactivating feature of the vector that enhances its biosafety. A CMV reporter that is located immediately upstream of the L_{10}-YFP sequence also drives foreign gene expression. (B) Lentivirus was generated by co-transfecting 293FT cells with L_{10}-YFP/pLenti, and ViraPower™ packaging mix containing pLP1, pLP2, and pLP/VSVG. The pLenti lacks important cis-acting elements needed for replication and transfer of the viral genome. The resulting virus particles are therefore replication-deficient, and the infection is limited to a single round without spreading (Dull et al, 1998). (C) Fluorescence image of 293FT cells 2 days following co-transfection with L_{10}-YFP/pLenti and ViraPower™ virus packaging mix. The fluorescence corresponds to YFP arising from expression of L_{10}-YFP. The white bar represents 5 µm.
Figure 2. Visualization of L10-YFP delivery to target cells using lentivirus. (A) Fluorescence image of Jurkat cells labeled by incubation with lentivirus that was collected from producer cells that were transfected as described in Figure 1. For labeling, 10⁶ cells were incubated with 100 µl of sonicated virus stock for 3 hr at 37°C. (B) Fluorescence (top) and bright field (bottom) images of Jurkat cells that were incubated with virus harvested from 293FT cells that were co-transfected with an empty pLenti that did not contain the L10-YFP sequence, and Virapower™ virus packaging mix. The white bars in (A) and (B) represent 5 µm. (C) L10-YFP labeling by lentivirus is independent of protein synthesis in target cells. The separate plots represent Jurkat cells that were either treated with 50 µg/ml cyclohexamide for 2 hr prior to addition to virus (red), or did not receive cyclohexamide (blue). In the treated sample, the cyclohexamide was maintained in the culture during incubation with the virus. Both samples were labeled by incubating with 100 µl of lentivirus for 3 hr at 37°C. The blue plot represents control Jurkat cells that did not receive cyclohexamide and were not incubated with virus. (D) Antibody to VSV G effectively blocks labeling of Jurkat cells by lentivirus. 10⁶ Jurkat cells were incubated with virus stock that was either untreated (+ Virus), or that was pre-incubated with a 1:100 dilution of sera to VSV (Rose and Bergmann, 1983) for 30 min at 37°C (+αVSV G, Virus). One sample received no virus, and served as a control for nonspecific fluorescence in the Jurkat cells. The mean relative fluorescence intensity (Mean Rel. Fl. Int.) of each sample was measured by flow cytometry.

Figure 3. Titration measurements of labeling of Jurkat cells by lentivirus. (A) 10⁶ cells were incubated with 0, 10, 20, 50, and 100 µl of lentivirus stock for 2 hr at 37°C, each in a final volume of 200 µl. (B) 10⁶ cells were incubated with 60 µl of virus in a final volume of 200 µl for 5 min, 30 min, 1 hr, 2 hr, and 4 hr. The fluorescence labeling of each sample in (A) and (B) was measured by flow cytometry.
C. Labeling by virus from producer cells expressing $L_{10}'$-YFP trans to pLenti

Figures 2 and 3 show that the plasmid pLenti containing $L_{10}'$-YFP functions to generate infectious particles that deliver the $L_{10}'$-YFP from producer cells to target cells. Next, to determine if virus from producer cells expressing $L_{10}'$-YFP trans to pLenti also labeled target cells, $L_{10}'$-YFP was expressed in 293FT cells using the vector pWay20 rather than pLenti. The pWay20 plasmid contains the $L_{10}'$-YFP gene under the transcriptional control of a CMV promoter (Rodgers, 2002).

The manner by which $L_{10}'$-YFP-loaded virus was produced by trans expression of $L_{10}'$-YFP is illustrated in Figure 4A. First, in order to ensure that expression of $L_{10}'$-YFP was sufficient to provide effective loading of the virions, 293FT cells were transfected with the pWay20 plasmid 24 hr prior to co-transfection with pLenti and the ViraPower™ packaging mix. Virus was then harvested 48 hr following the second transfection. As shown in Figure 4B, lentivirus generated by trans expression of $L_{10}'$-YFP in producer cells was also able to effectively label target cells.

D. Lentivirus-dependent labeling of cells does not disrupt cell stimulation

One concern was that the viral load that was required for detectable labeling of the target cells would be disruptive to the cells. For example, delivery of foreign proteins by lentivirus could be accompanied by cell cytotoxicity that is associated with incubating cells with relatively high titers of virus for several hours. To test for this possibility, we compared the response of labeled and unlabeled Jurkat cells to stimulation by crosslinking the T cell receptor (TCR). The response to stimulation was assayed by measuring protein phosphotyrosine signals in whole cell lysates. As shown in Figure 5, a control sample that did not receive virus demonstrated a robust increase in the tyrosine phosphorylation of multiple proteins following stimulation, as did the sample that was labeled using lentivirus prior to stimulation. Thus, labeling Jurkat cells using lentivirus was not deleterious to the cells as measured by their response to TCR-dependent signaling.

![Figure 4](image-url)
IV. Discussion

Lentivirus systems using HIV-based expression vectors are a developing tool in gene therapy. These systems use an HIV-derived machinery to assemble and bud viruses, but are genetically altered from the wild type HIV so as to increase their biosafety (Poznansky et al., 1991; Shimada et al., 1991; Naldini et al., 1996; Reiser et al., 1996; Dull et al., 1998). One feature of HIV is that its assembly and budding occurs in glycolipid-enriched membrane rafts within cell membranes (Nguyen and Rildreth, 2000; Ono and Freed, 2001; Manes et al., 2003). Membrane rafts are a ubiquitous class of membrane domains which function in cell signaling, protein trafficking, and pathogen entry and exit from cells (Brown and London, 1998; Simons and Toomre, 2000; Manes et al., 2003) (Helms and Zurzolo, 2004; Rodgers et al., 2005; Rodgers and Smith, 2005). In the case of lentiviruses, rafts may function as a scaffold for assembly of the virus particles. Rafts may also represent an optimal point for budding of nascent virions from the plasma membrane. For example, studies have shown that separate phase regions of membranes, including those enriched with rafts, have a larger curvature than the remaining bilayer (Baumgart et al., 2003; Bacia et al., 2005), and this may function to augment membrane budding in cell membranes.

Using one example of an HIV-derived lentivirus expression vector, we have shown here that constitutive overexpression of a raft-associated protein in producer cells resulted in its efficient delivery to target cells. Labeling of target cells by virus was specific to virus-dependent fusion to the target cell, as it was effectively blocked by pre-incubating the virus stock with antibody to the surface glycoprotein of the virus that was produced for this study. Also, protein that was delivered to the target cells appeared to become incorporated in the same cellular pools as that produced by gene expression. This latter observation underscores the potential utility of cell labeling by viral delivery, such that a foreign protein can be delivered to discrete pools within cells through the appropriate targeting signals.

One possible mechanism for transfer of L\textsubscript{10}-YFP to target cells through lentivirus is by loading of the L\textsubscript{10}-YFP into the viral envelope during assembly and budding of the virus. The loaded protein could then be released into the cell membrane pools following fusion of the virus to the target cell. One explanation for the efficient labeling achieved with L\textsubscript{10}-YFP is that, as a raft-associated protein, it is preferentially incorporated into the viral envelope. It follows that proteins that are excluded from rafts will be incorporated into the virions less efficiently than proteins such as L\textsubscript{10}-YFP. Consistent with this hypothesis, we found that labeling of target cells with lentivirus produced from cells expressing the construct S\textsubscript{15}-YFP, which is excluded from rafts, was less efficient than the labeling by an equivalent titer of the L\textsubscript{10}-YFP/lentivirus (data not shown).

Another factor besides raft-association that is likely to be a determinant regarding the efficiency by which the targeted protein is loaded into the virus is the size of the foreign protein. We are currently exploring the molecular weight cut off for virus loading and delivery to target cells, and whether this system is amendable for fusion proteins other than GFP. One outcome of these studies may...
therefore be evidence that lentivirus can be used to efficiently transfer enzymatic activity to target cells for therapeutic purposes.

One concern with using the lentivirus as a protein delivery device is the biohazard associated with this class of viruses. The system employed in this study is a third generation lentivirus vector that contains a number of safeguards that eliminate the possibility of production of replication-competent virions (Dull et al, 1998). These include usage of a limited set of the HIV genes, which are expressed trans on non-overlapping plasmids, thereby guarding against generation of productive recombinants.

In summary, we have described a facile method for delivering foreign proteins to target cells using a lentivirus plasmid that was originally developed for gene delivery. Our findings suggest that protein delivery occurs by generation of loaded virus through constitutive expression of the foreign protein in producer cells. The current findings therefore demonstrate a novel strategy for labeling rafts in cells, and future studies may demonstrate application of this technology for delivery of enzymes to offset certain pathologies.

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References


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