AMERICAN SOCIETY of
GENE & CELL
THERAPY

Improved GaLV-TR Glycoproteins to Pseudotype Lentiviral Vectors: Impact of Viral Protease Activity in the Production of LV Pseudotypes

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Lentiviral vectors (LVs) are excellent tools for gene transfer into mammalian cells. It is noteworthy that the first gene therapy treatment using LVs was approved for commercialization in 2017. The G glycoprotein from rhabdovirus vesicular stomatitis virus (VSV-G) is the glycoprotein most used to pseudotype LVs, due to its high efficiency in transducing several cell types and its resistance to viral vector purification and storage conditions. However, VSV-G expression induces cytotoxicity, which limits LV production to short periods. As alternative to VSV-G, γ-retrovirus glycoproteins (4070A derived, GaLV derived, and RD114 derived) have been used to pseudotype both γ-retroviral vectors (RVs) and LVs. These glycoproteins do not induce cytotoxicity, allowing the development of stable LV producer cells. Additionally, these LV pseudotypes present higher transduction efficiencies of hematopoietic stem cells when compared to VSV-G. Here, new 4070A-, RD114-TR-, and GaLV-TR-derived glycoproteins were developed with the aim of improving its cytoplasmic tail R-peptide cleavage and thus increase LV infectious titers. The new glycoproteins were tested in transient LV production using the wild-type or the less active T26S HIV-1 protease. The GaLV-TR-derived glycoproteins were able to overcome titer differences observed between LV production using wild-type and T26S protease. Additionally, these glycoproteins were even able to increase LV titers, evidencing its potential as an alternative glycoprotein to pseudotype LVs.

INTRODUCTION

Retroviral vectors can deliver and integrate a gene of interest into a target cell genome upon cell transduction, allowing a long-term constitutive expression. In the particular case of lentiviral vectors (LVs), these are able to promote gene transfer into both dividing and non-dividing cells,[1] potentially presenting lower genotoxicity than γ-retroviral vectors (γ-RVs) due to their integration pattern.[2,3] These reasons justify the growing number of gene therapy clinical trials using LVs in the past 2 decades with the aim of treating several disorders.[4]

LVs may be pseudotyped with heterologous viral glycoproteins acquiring alternative tropism and specific cell-entry properties.[5,6] Generally, the G glycoprotein from rhabdovirus vesicular stomatitis virus (VSV-G) is used to pseudotype LVs since, the wide distribution and high molecule number of VSV-G receptors at the cell surface allow for efficient transduction of several cell types.[7–9] Additionally, LVs pseudotyped with VSV-G can be concentrated by ultracentrifugation without a substantial loss of infectivity and are resistant to several freeze-thaw cycles, which also contributes for their wide usage.[6] However, the syncytium formation and consequent cytotoxicity, both resultant from VSV-G fusogenicity activation in viral producer cells, do not allow LV production for more than a few days.[10–12] The VSV-G-pseudotyped LVs are usually produced transiently by cotransfection of 293T cells with LV expression cassettes or constitutively produced by LV producer stable cell lines making use of inducible promoters to drive VSV-G expression.[10–12]

As alternatives to VSV-G, the amphotropic murine leukemia virus (4070A), gibbon ape leukemia virus (GaLV), and feline endogenous retrovirus (RD114) glycoproteins have been used to pseudotype both γ-RVs and LVs.[13–22] The production of γ-RVs pseudotyped with these three γ-retrovirus glycoproteins do not present major constraints. However, while the production of LVs pseudotyped with 4070A has no impact on infectious titers,[23–27] the co-expression of HIV-1 core proteins with GaLV or RD114 glycoproteins barely results in the production of infectious LVs.[28–32] These titer asymmetries are due to differences between cytoplasmatic tail amino-acid sequences of the glycoproteins (Figure 1A), since the fully fusogenic activation of 4070A, RD114, and GaLV is dependent on the cytoplasmatic tail R-peptide recognition and cleavage by the viral protease.[33–42] The replacement of the RD114 and GaLV cytoplasmic tail region by the one for 4070A (originating the RD114-TR and GaLV-TR, respectively) allows

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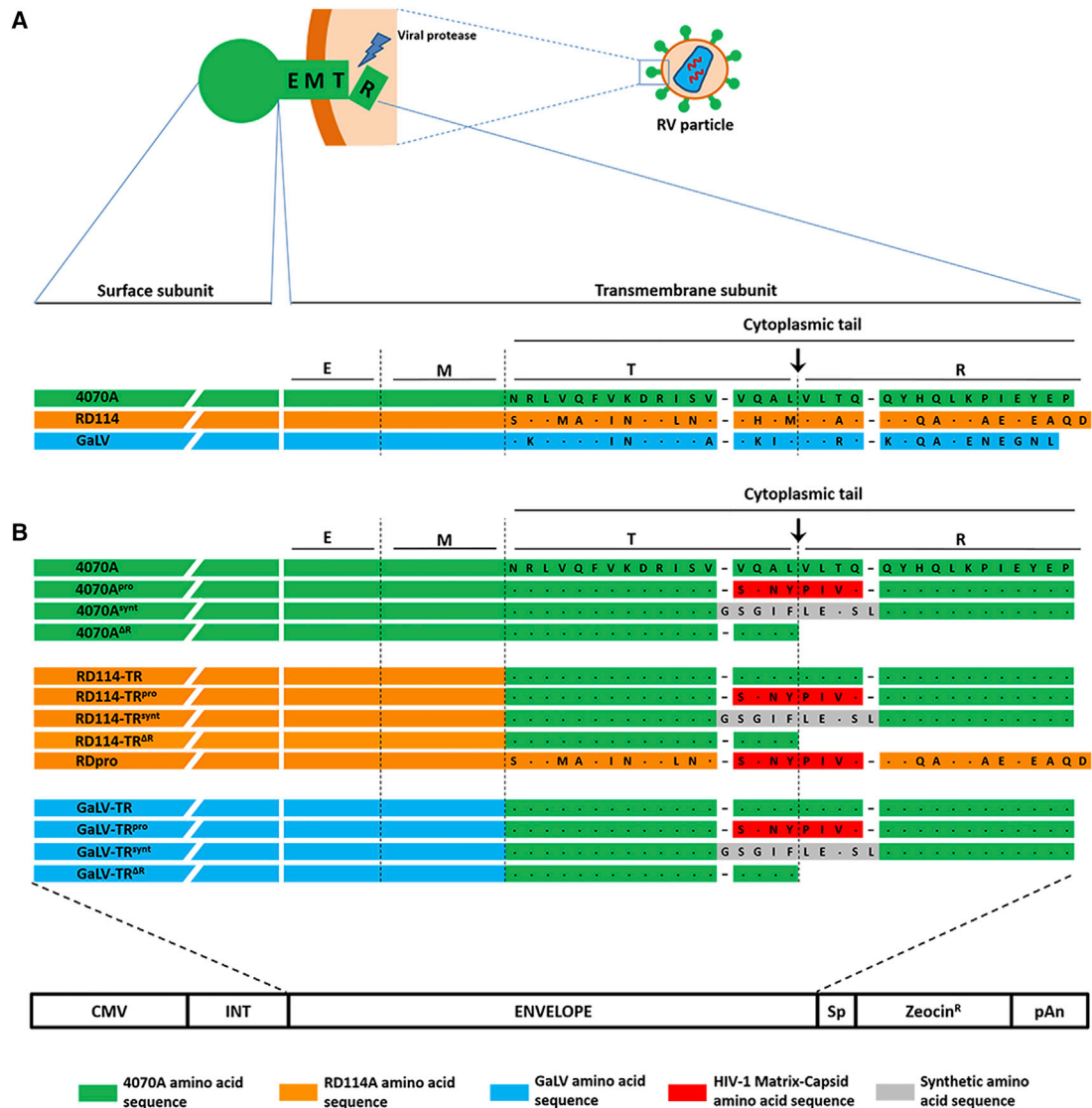
Figure 1. Schematic Representation of Wild-Type and Engineered Viral Envelope Glycoproteins

(A) Amino-acid sequence alignment of glycoprotein cytoplasmic tail: murine leukemia virus (4070A), endogenous feline retrovirus (RD114), and gibbon ape leukemia virus SEATO strain (GaLV). (B) Amino-acid sequence alignment of the cytoplasmic tail region of the glycoproteins used in LV production and schematic representation of the envelope expression cassette. The black arrows indicate the protease cleavage site. E, ectodomain; M, transmembrane domain; T, cytoplasmic tail after R-peptide cleavage; R, R-peptide; CMV, cytomegalovirus promoter; INT, intron; Sp, spacer; pAn, polyadenylation site.

a high increase of LV particle infectivity.[19,23,28,29,31,32] Alternatively, just by replacing the protease cleavage sequence of RD114 with a sequence naturally cleaved by the HIV-1 protease—namely, the matrix-capsid cleavage sequence of HIV-1 Gag-Pro-Pol polyprotein (originating the RDpro glycoprotein)—it is also possible to increase the production of infectious LV particles.[23,30,43]

The LVs pseudotyped with the γ-retrovirus glycoproteins are able to transduce several cell types, being particularly efficient in the transduction of progenitor and differentiated hematopoietic stem cells (HSCs), when compared to the VSV-G pseudotype.[19–22,28] Moreover, these LV pseudotypes are not inactivated by human serum[23,28] and may be concentrated by ultrafiltration, ultracentrifugation, or tangential flow filtration.[20,44–46] Additionally, the fusogenic activation of these glycoproteins should mainly occur after being incorporated into the viral particles (during viral maturation, when the viral particle buds out of the cell), preventing syncytium formation, which allows the development of stable cell lines able to constitutively produce LVs for several weeks.[43,47–49] Recently, we have established a stable LV producer cell line using a Gag-Pro-Pol polyprotein with a less active HIV-1 protease and the 4070A glycoprotein, able to constitutively produce 10^6 transducing units (TUs) per milliliter (TU · mL^{-1}) for at least 2 months.[50]

The main drawback of pseudotyping LVs with γ-retrovirus glycoproteins is related to its lower infectious titers when compared to VSV-G pseudotype.[20,23,30,31] In this work, new versions of 4070A, GaLV-TR, and RD114-TR glycoproteins with modified sequences on the cytoplasmic tail were developed with the aim of improving glycoprotein fusogenicity activation and, consequently, increasing infectious LV titers. The impact of the modified glycoproteins in viral titers was assessed by performing transient LV production using either the wild-type or the mutated less active T26S HIV-1 protease.

RESULTS

Modifications on γ-Retrovirus Glycoprotein Cytoplasmic Tail

The amino-acid protease cleavage sequence VQALVLTQ on the cytoplasmic tail of 4070A, RD114-TR, and GaLV-TR glycoproteins was replaced by the HIV-1 natural sequence SQNYPIVQ[51] or the synthetic sequence GSGIFLETSL,[52] originating the *glycoprotein^{pro}* and *glycoprotein^{synt}* modified versions, respectively (Figure 1B). A truncated version of each glycoprotein with the R-peptide sequence deleted was also developed, the *glycoprotein^{ΔR}*, to be used as cleaved glycoprotein positive control. All the envelope glycoprotein genes were introduced in the same expression cassette to allow direct comparison between the different LV pseudotype productions (Figure 1B).

Transient Productions of LV Pseudotypes Using the Wild-Type Viral Protease

Transient production of third-generation SIN-LVs (self-inactivating lentiviral vectors) was performed for each pseudotype using the *gag-pro-pol* coding the wild-type HIV-1 protease. The supernatant titers (Transduction Units [TUs] and physical particles [PPs]) were assessed and compared (Figure 2A). The LV production of 4070A and RD114-TR pseudotypes generated average titers of about 1.2×10^7 TU $\cdot$ mL^{-1} and 1.5×10^6 TU $\cdot$ mL^{-1}, respectively. The new *glycoprotein^{pro}* and *glycoprotein^{synt}* versions of 4070A and RD114-TR did not markedly alter LV titers. However, a decrease in infectious titers was detected for production using 4070A^{ΔR} and RD114-TR^{ΔR}, leading to a 3-fold and 6-fold decrease in the TU/PP ratio when compared to the production of LVs pseudotyped with the parental 4070A and RD114-TR, respectively. In addition to RD114-derived production, the RDpro glycoprotein was also used, leading to a 3-fold decrease in the TU/PP ratio, when compared to RD114-TR production. The LV production of GaLV-TR pseudotype generated a titer of about 1.3×10^6 TU $\cdot$ mL^{-1}. In the case of GaLV-TR-derived glycoproteins, while the GaLV-TR^{pro} did not change LV titers, a 2- and 5-fold increment on functional LV titers were observed for the LV production using GaLV-TR^{synt} and GaLV-TR^{ΔR} glycoproteins, respectively, generating a maximum average titer of about 6.1×10^6 TU $\cdot$ mL^{-1}. The LV production of VSV-G pseudotype generated an average titer of about 2.8×10^7 TU $\cdot$ mL^{-1}.

Transient Productions of LV Pseudotypes Using the Less Active T26S Viral Protease

LV production, identical to that described earlier, was also performed using the *gag-pro-pol* with the T26S mutated protease (Figure 2B). For the viral production of 4070A-derived, RD114-derived, and VSV-G LV pseudotypes, average titer values similar to the ones previously observed were detected. However, for the LV production of GaLV-TR pseudotype, an average infectious titer of just 1.5×10^5 TU.mL^{-1} was observed. Nevertheless, infectious titer increases of 9-, 16-, and 35-fold were observed for GaLV-TR^{pro}, GaLV-TR^{synt}, and GaLV-TR^{ΔR} LV-pseudotype production, respectively, generating a maximum average titer of about 5.3×10^6 TU $\cdot$ mL^{-1}.

Syncytium Formation on 293T Cells Transiently Expressing the Viral Envelope Glycoproteins
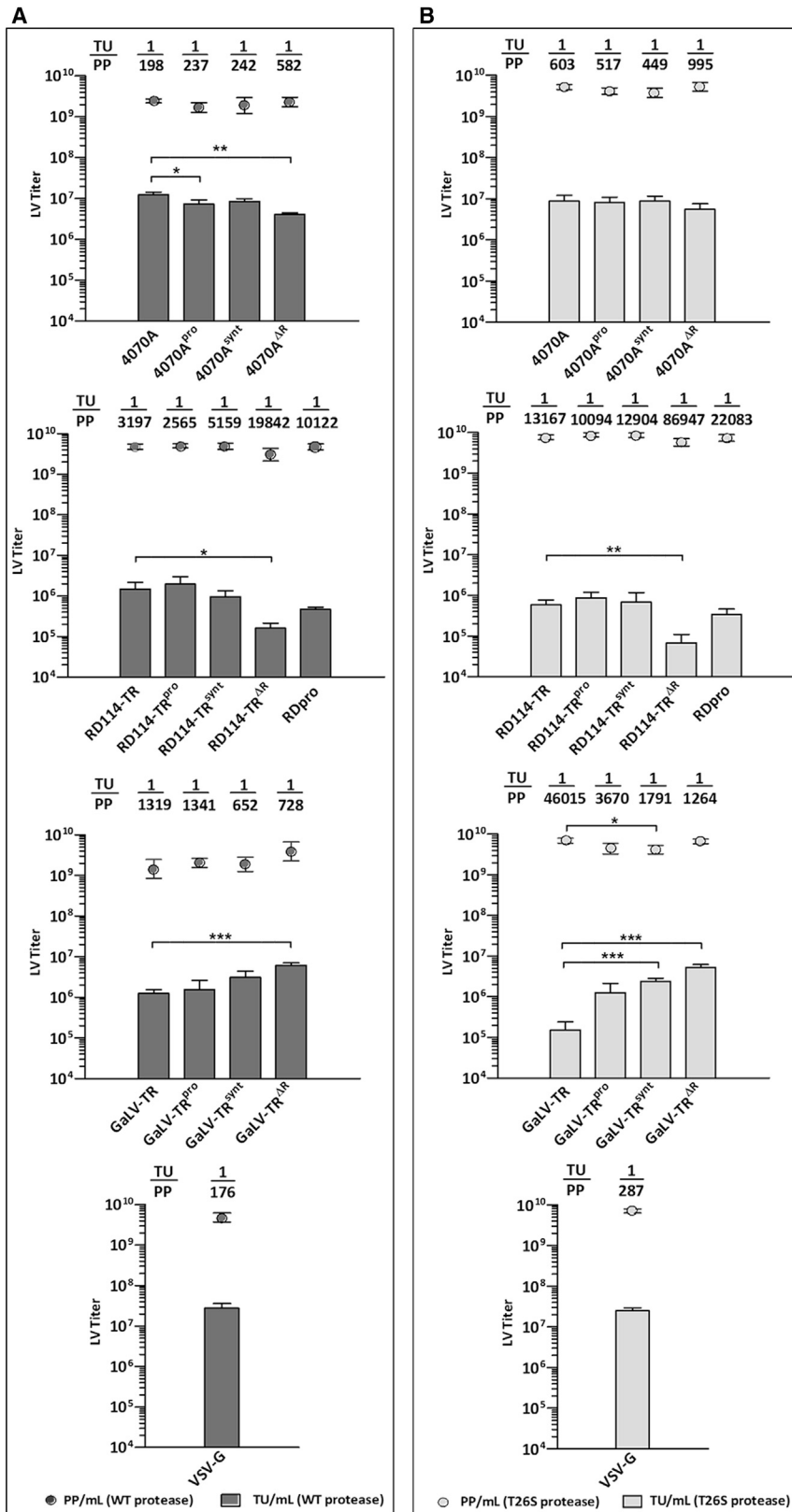
Syncytium formation induced by glycoprotein expression was evaluated in 293T cells transiently transfected with the plasmids coding for envelope glycoproteins. 24 h post-transfection, cells were observed by phase-contrast microscopy (Figure 3). Syncytium and non-adherent round cells were observed in cells transfected with RD114-TR^{ΔR}, GaLV-TR^{ΔR}, and VSV-G expression cassettes. Additionally, RD114-TR^{pro} expression also led to the formation of few syncytia. In all the other cases, no major morphological cell differences, relative to the no expression control, were observed.
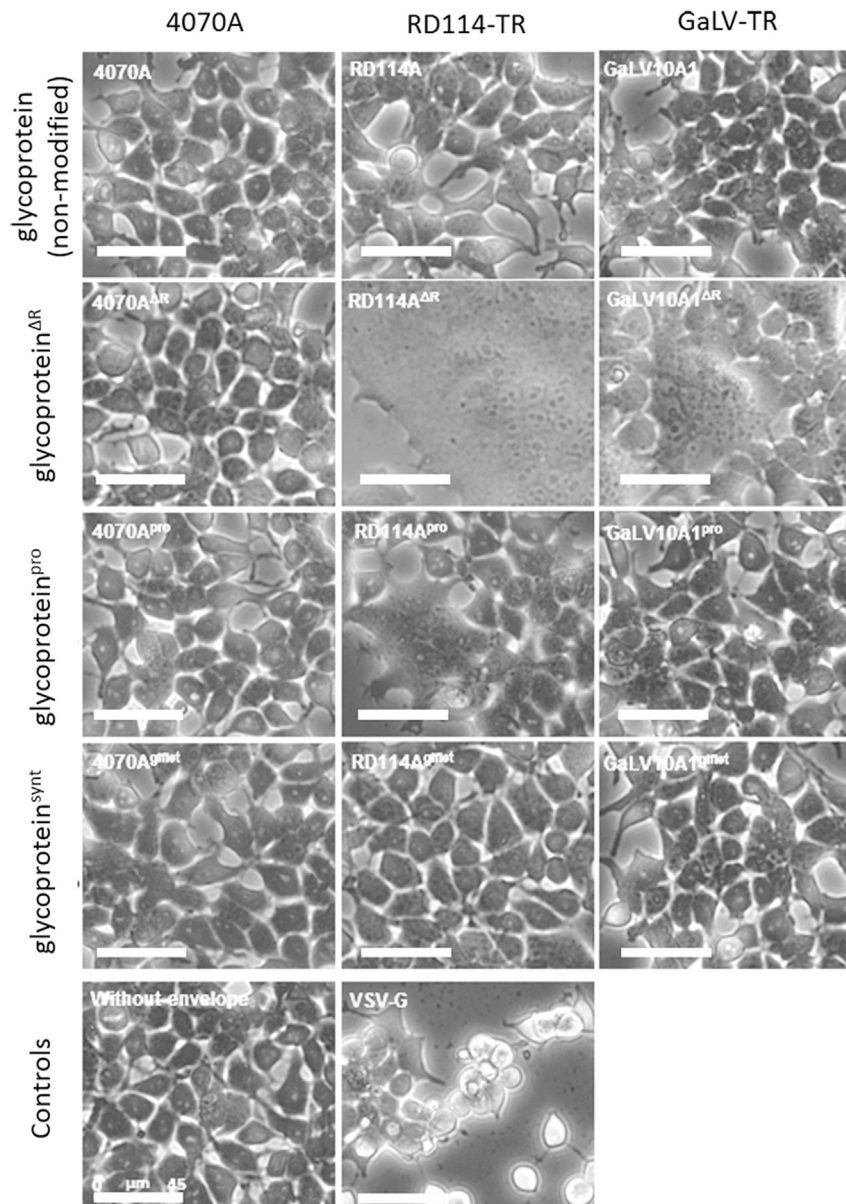
DISCUSSION

The 4070A-, RD114-, and GaLV-derived envelope glycoproteins allow an efficient LV transduction of hematopoietic stem cells, conferring a more restricted tropism to LVs than VSV-G.[19–22,28] Additionally, these glycoproteins can be used as an alternative to VSV-G in the development of stable constitutive LV producer cell lines since they are non-cytotoxic.[43,47–49] Despite the advantages of γ-retrovirus glycoproteins, the production of LVs pseudotyped with these usually present lower infectious titer particles when compared to the production of LVs pseudotyped with VSV-G.

To evaluate whether improved cleavage of the R-peptide in 4070A, RD114-TR, and GaLV-TR glycoproteins would translate into increased infectious titers of those LVs pseudotypes, the protease recognition sequence on the cytoplasmic tail of these glycoproteins was replaced by two others described to be efficiently cleaved by the HIV-1 protease: (1) the matrix-capsid natural cleavage sequence SQNYPIVQ, which is present in the HIV-1 Gag-Pro-Pol polyprotein,[51] and (2) the GSGIFLETSL synthetic peptide.[52] A truncated version of each glycoprotein without R-peptide was also developed. These truncated glycoproteins should be produced in their fully fusogenic states; thus, it would be expected that the resultant particles should exhibit the maximum infectious titer for each specific pseudotype (if no major interference on glycoprotein cellular traffic and assembling occurs).

The titer analysis of LV production using the wild-type protease evidences that the engineered 4070A-derived and RD114-TR-derived glycoproteins did not markedly alter LV titers (Figure 2A). Furthermore, the respective truncated glycoprotein versions (4070A^{ΔR} and RD114-TR^{ΔR}) impaired the production of infectious LVs. Nevertheless, the results of RD114-TR and RDpro LV production clearly indicate that RD114-TR is preferable to RDpro for producing higher titers of infectious LVs. In contrast to 4070A-derived and RD114-TR-derived glycoproteins, a 3- to 5-fold improvement in titers of

Figure 2. Transient LV Production Titers with the Engineered Envelope Glycoproteins

(A and B) Transient production titers of LVs pseudotyped with 4070A, 4070A-derived, RD114-TR, RD114-TR-derived, RDpro, GaLV-TR, GaLV-TR-derived, or VSV-G glycoproteins using the (A) HIV-1 wild-type protease or (B) HIV-1 T26S protease. The bars represent the transducing units (TUs), and the circles represent the physical particles (PPs). The titer values presented are the means ± SD of 3 independent experiments (n = 3). Statistical analysis for the comparison of LV titers was performed by using an unpaired Student t test (two-tailed). *p < 0.05; **p < 0.01; ***p < 0.001. The ratios of TUs to PPs are indicated above each set of LV titers.

Figure 3. Representative Pictures of HEK293T Cells Transiently Expressing Envelope Glycoproteins

80× bright-field microscopy. Scale bars, 45 μm.

In the specific case of the R-peptide-truncated glycoproteins developed herein, these are produced in a fusogenic active state. Thus, once at the cell-membrane surface, the glycoproteins will interact with the respective cell receptors of neighboring cells, promoting cell membrane fusion and consequent syncytia formation.[29,31,41] This may explain the large syncytia observed in cells transfected with RD114-TR$^{\Delta R}$ and GaLV-TR$^{\Delta R}$ (Figure 3) and in the respective LV production. Nonetheless, unlike for RD114-TR$^{\Delta R}$, the syncytia on GaLV-TR$^{\Delta R}$ LV production did not negatively affect LV titers (Figure 2A).

The impact of protease activity in the production of several LV pseudotypes was also evaluated by assessing LV titers obtained from transient LV production using the HIV-1 *gag-pro-pol* gene with the less active T26S mutated protease.[55] Comparing the 4070A, RD114-TR, and GaLV-TR LV production titers of both proteases, it is possible to observe that the lower activity of T26S protease affects the production of infectious LVs. Whereas the less active protease led to merely a 3- to 4-fold decrease in TU/PP ratio for 4070A and RD114-TR pseudotypes, a 35-fold ratio decrease was observed with the GaLV-TR pseudotype. This decrease was mainly due to the 10-fold reduction of infectious GaLV-TR LVs when using the T26S protease. Nevertheless, the modified glycoproteins GaLV-TR^{pro}, GaLV-TR^{synt}, and GaLV-TR$^{\Delta R}$ allowed the rescue of LV titers to levels identical to those obtained in production using the wild-type protease. As expected, the production titers of LVs pseudotyped with VSV-G were not affected by the T26S protease, since this glycoprotein is not dependent on viral protease cleavage to become fusogenically active.[56] Together, these results demonstrate that the lower activity of T26S protease, despite not affecting Gag-Pro-Pol production and its respective viral processing, impairs the R-peptide cleavage of 4070A, RD114-TR, and GaLV-TR glycoproteins, which directly affects the infectivity of LVs. Additionally, since titers of these three LV pseudotypes were not similarly affected, despite their common cytoplasmic tail (Figure 1B), it is suggested that the glycoprotein sequence upstream regions (ectodomain + transmembrane domain of transmembrane subunit and surface subunit) may condition R-peptide recognition and cleavage by the viral protease. We hypothesize that the amino-acid differences on those glycoprotein upstream regions may promote different

infectious LVs in relation to the parental glycoprotein (GaLV-TR) was observed when using the GaLV-TR^{synt} and GaLV-TR$^{\Delta R}$ glycoproteins, leading to a 2-fold increase in the TU/PP ratio. These results suggest that, unlike for 4070A and RD114-TR pseudotypes, the infectivity of GaLV-TR vectors is limited by an inefficient HIV-1 protease cleavage of the glycoprotein R-peptide.

The R-peptide cleavage by the viral protease and consequent glycoprotein fusogenic activation should mainly occur during viral particle maturation, at the viral budding step (outside of the cell).[53,54] This should allow cellular glycoprotein production, intracellular trafficking, and cell membrane incorporation in its non-fusogenic conformation, preventing counterproductive cell interactions.[34–37]

conformational changes in the cytoplasmic tail of 4070A, RD114-TR, and GaLV-TR glycoproteins, hampering the accessibility of viral protease to the respective cleavage sequences.

In this work, new GaLV-TR-derived glycoproteins were developed to pseudotype LVs. Those glycoproteins allowed us to transiently produce a maximum LV titer of 6.0×10^6 TU · mL^{-1}, surpassing the titers of LV pseudotypes with GaLV-TR, RD114-TR, or RDpro. Additionally, this work evidences that glycoprotein R-peptide cleavage efficiency by viral protease limits titers of infectious LVs. The new GaLV-TR^{synt} and GaLV-TR^{pro} are potential alternatives to both VSV-G or RD114-derived glycoproteins for the establishment of constitutively stable LV producer cell lines. Although further studies are required, these novel envelopes show potential for the development of alternative high-titer viral vector production platforms.

MATERIALS AND METHODS

Cell Culture

HEK293T cells (CRL-11268), obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), were used for LV production and titration. Cells were cultured in DMEM (GIBCO, Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO). Cells were maintained in an incubator at 37°C with a humidified atmosphere of 7% CO_2 in air. The trypan blue exclusion method was used to assess cell concentration and viability.

Plasmids

Plasmid pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene #12252, kindly provided by Dr. Didier Trono) codes for a SIN-LV genome carrying a GFP as a reporter gene and was used as a transgene in all LV production.

Plasmid pMDLg/pRRE[57] (Addgene #12251, kindly provided by Dr. Didier Trono) codes for HIV-1 Gag-Pro-Pol polyprotein. The introduction of the T26S point mutation on the viral protease sequence originated the pGP(T26S)P plasmid.[50] Plasmid pRSV-REV[57] (Addgene #12253, kindly provided by Dr. Didier Trono) codes for the second and third exons of HIV-1 *rev*. These plasmids coding for the LV packaging functions were used in transient LV production.

Plasmids coding for envelope glycoproteins are as follows: pMD2.G (Addgene #12259, kindly provided by Dr. Didier Trono) codes for VSV-G; pCMV-GaLV-TR codes for a modified glycoprotein of the GaLV SEATO strain and results from the removal of 19 nt prior to the start codon of the GaLV glycoprotein from phGaLV10A1 by inverse PCR; phGaLV10A1 was kindly provided by Dr. Otto Merten (Généthon, Évry, France); pCMV-RD114-TR codes for a modified RD114 glycoprotein, amplified from the pLTR-RD114A[19] plasmid (Addgene #17576, kindly provided by Dr. Jakob Reiser) and cloned into the vector resultant from phGaLV10A1 restriction with EcoRI and KasI enzymes; pCMV-RDpro codes for RDpro[43] glycoprotein, which was chemically synthesized (GeneScript, Piscataway, NJ, USA) and cloned into the vector resultant from phGaLV10A1 restriction with EcoRI and KasI enzymes; pCMV-4070A codes for the ampho-

tropic MLV glycoprotein amplified from pMonoZeo-4070A[50] and cloned into the vector resultant from phGaLV10A1 restriction with EcoRI and KasI enzymes; pCMV-GaLV-TR^{pro}, pCMV-RD114-TR^{pro}, and pCMV-4070A^{pro} code for the respective modified viral glycoproteins in which the viral protease cleavage sequence VQALVLTQ of the 4070A glycoprotein cytoplasmic tail was replaced by that of the HIV-1 Gag matrix-capsid, SQNYPIVQ, by inverse PCR from the parental plasmids; pCMV-GaLV-TR^{synt}, pCMV-RD114-TR^{synt}, and pCMV-4070A^{synt} code for the respective viral modified glycoproteins in which the viral protease cleavage sequence VQALVLTQ of the 4070A glycoprotein cytoplasmic tail was replaced by synthetic peptide GSGIFLETSL[52] by performing two inverse PCRs from the parental plasmids; pCMV-GaLV-TR$^{\Delta R}$, pCMV-RD114-TR$^{\Delta R}$, and pCMV-4070A$^{\Delta R}$ code for the respective truncated glycoproteins in which the R-peptide was deleted from the cytoplasmic tail of the glycoproteins and replaced by a STOP codon by inverse PCR.

The primers and templates used in plasmid constructions, as well as the cloning strategies, are described in Table S1.

Transient LV Production

Transient LV production was performed by transfecting 293T cells as described by Tomás et al. (2018).[50] Briefly, 6×10^4 cells per square centimeter were seeded in tissue culture flasks and, 24 hours later, transfected using linear 25 kDa polyethyleneimine (PEI; Polysciences, Hirschberg an der Bergstrasse, Germany) at a mass ratio of 1:1.5 (DNA:PEI), with the respective plasmids. The amount of each viral component per million cells was the following: 2.5 μg vector genome; 1 μg Gag-Pro-Pol; 0.25 μg Rev; 0.9 μg envelope. Medium was exchanged 24 hours after transfection. 24 hours after medium exchange, the supernatant was collected, clarified at 0.45 μm, and stored at −80°C.

Transducing LV Particle Titers

The transducing LV titer determination was performed by transducing 293T cells with the produced supernatants followed by flow cytometry analysis for GFP expression, as described by Tomás et al. (2018).[50] The concentration of LV TUs (TU · mL^{-1}) was calculated using the following equation:

$$\text{Titer}\left(\frac{\text{TU}}{\text{mL}}\right) = \frac{\% \text{ of GFP positive cells}}{100} \times \frac{\text{dilution factor}}{\text{volume of transduction}}$$
$$\times \text{ n}° \text{ of cells at transduction time.}$$

Physical LV Particle Titers

The concentration of p24 LV protein in supernatants was determined by a p24 ELISA using the INNOTEST HIV Antigen mAb (Fujirebio Diagnostics, Malvern, PA, USA), following the manufacturer's instructions. It was assumed that 1 ng p24 corresponds to 1.25×10^7 physical particles.[58] The titer of LV PPs (PP · mL^{-1}) was estimated using the following equation:

$$\text{Titer}\left(\frac{\text{PP}}{\text{mL}}\right) = [\text{p24 protein}] \times 12500000.$$

Statistical Analysis

To assess the significance of differences seen among titers of LV-pseudotype production, statistical analysis was used to evaluate data from multiple experiments using GraphPad Prism v5 (GraphPad Software, San Diego, CA, USA). For the comparison of LV titers differences on p values <0.05, using unpaired Student t test (two-tailed), were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.08.001.

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