Virus Research 153 (2010) 29–35

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Development of a lentiviral vector system to study the role of the Andes virus glycoproteins

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ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 24 June 2010
Accepted 1 July 2010
Available online 7 July 2010

Keywords:
Bunyavirus
Pseudotyped particle
Glycoprotein function
Inhibition

ABSTRACT

To infect target cells, enveloped viruses use their virion surface proteins to direct cell attachment and subsequent entry via virus-cell membrane fusion. How hantaviruses enter cells has been largely unexplored. To study early steps of Andes virus (ANDV) cell infection, a lentiviral vector system was developed based on a Simian immunodeficiency virus (SIV) vector pseudotyped with the ANDV-Gn/Gc envelope glycoproteins. The incorporation of Gn and Gc onto SIV-derived vector particles was assessed using newly generated monoclonal antibodies against ANDV glycoproteins. In addition, sera of ANDV infected humans were able to block cell entry of the SIV vector pseudotyped with ANDV glycoproteins, suggesting that their antigenic conformation is similar to that in the native virus. The use of such SIV vector pseudotyped with ANDV-Gn/Gc glycoproteins should facilitate studies on ANDV cell entry. Along this line, it was found that depletion of cholesterol from target cells strongly diminished cell infection, indicating a possible role of lipid rafts in ANDV cell entry. The Gn/Gc pseudotyped SIV vector has several advantages, notably high titer vector production and easy quantification of cell infection by monitoring GFP reporter gene expression by flow cytometry. Such pseudotyped SIV vectors can be used to identify functional domains in the Gn/Gc glycoproteins and to screen for potential hantavirus cell entry inhibitors.

1. Introduction

ANDV is a human pathogen that causes a pulmonary syndrome with mortality rates of up to 40%. This virus belongs to the *Hantavirus* genus and was discovered in 1995 in Argentina (Lopez et al., 1996). In contrast to other hantaviruses, ANDV causes a lethal disease in an animal model (Hooper et al., 2001) which gained a strong interest to study the ANDV associated pathology as well as for the development of treatment and prevention strategies.

Hantaviruses represent one of five genera of the *Bunyaviridae* family and are characterized by a tripartite ssRNA(−) genome that encodes at least four proteins: the RNA-dependent RNA polymerase, nucleoprotein and Gn and Gc glycoproteins (Elliott, 1990). Transmission of hantaviruses to humans occurs via inhalation of contaminated aerosolized feces from rodents (Nuzum et al., 1988), and for ANDV person-to-person transmission has also been proposed (Padula et al., 1998). To infect cells, hantaviruses interact with specific receptors (Choi et al., 2008; Gavrilovskaya et al., 1998; Krautkramer and Zeier, 2008; Mou et al., 2006) followed by endocytosis (Jin et al., 2002). From within endosomes at low pH, hantaviruses are thought to fuse their envelope membrane with the membrane of endosomes, thereby releasing their ribonucleocapsids through a fusion pore into the cell cytoplasm where viral replication takes place (Arikawa et al., 1985; McCaughey et al., 1999).

The cascade of entry events is thought to be mediated by the viral Gn and Gc glycoproteins (Lundkvist et al., 1993; Ogino et al., 2004; Ruo et al., 1991), which are anchored into the virion envelope. The glycoproteins are derived from a common protein precursor which is co-translationally cleaved (Lober et al., 2001). Whilst the role of Gn in this process has not yet been resolved, it has been suggested that Gc corresponds to the active fusion protein of hantaviruses (Tischler et al., 2005b) and other bunyaviruses (Plassmeyer et al., 2005; Plassmeyer et al., 2007; Shi et al., 2009).

Efforts aimed at studying the functions of the hantavirus glycoproteins during the initial steps of the replication cycle have been hampered in part by the lack of a reverse genetic system that allows the generation of recombinant, infectious viruses. In addi-

Abbreviations: ANDV, Andes virus; CMV, Cytomegalovirus; HIV, Human immunodeficiency virus; GFP, green fluorescent protein; GPC, glycoprotein precursor; mβCD, methyl-β-cyclodextrin; RT, reverse transcriptase; SIV, Simian immunodeficiency virus; VSV, Vesicular stomatitis virus.

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doi:10.1016/j.virusres.2010.07.001

tion, infectious hantaviruses are replicating to only low titers *ex vivo* which also requires laboratories with a high biosafety level.

Some enveloped viruses such as retroviruses and rhabdoviruses can incorporate heterologous viral glycoproteins during budding into their envelope, giving rise to pseudotyped viral particles (Schnell et al., 1996; Soneoka et al., 1995; Suomalainen and Garoff, 1994). The pseudotyped particles have been used to characterize virus-cell entry in the case of different viruses, including pathogenic viruses such as filoviruses and arenaviruses (Flint et al., 2004; Garbutt et al., 2004; Larson et al., 2008; Takada et al., 1997). In this context, successful pseudotyping of viruses from the *Bunyaviridae* family has been reported for La Crosse virus and Hantaan virus using a retroviral derived system (Ma et al., 1999) and for Hantaan virus and Seoul virus using a Vesicular stomatitis virus (VSV) derived system (Lee et al., 2006; Ogino et al., 2003). Very recently, the VSV derived system has also been used to produce ANDV pseudotypes (Ray et al., 2010).

Here an alternative ANDV pseudotyping based on lentiviral vectors was developed which has the advantage to integrate a reporter gene into the host cell genome and consequently these vectors are suitable for studies in animals. The SIV-derived particles pseudotyped with ANDV-Gn/Gc glycoproteins have antigenic surface determinants of the wild type virus since patient sera can efficiently bind and neutralize the pseudotyped particles. Using the ANDV-Gn/Gc pseudotyped SIV particles (ANDV pseudotyped particles) for cell infection, ANDV cell tropism and its dependence on cholesterol for cell entry could be defined, revealing the value of these SIV based ANDV pseudotyped particles for *in vitro* and *in vivo* studies on ANDV cell entry and its inhibition.

2. Materials and methods

2.1. Cells and antibodies

Vero E6 cells (ATCC, CRL 1586), HeLa-P4 cells (ECACC), 293T (kindly provided by Généthon) and 293FT cells (Invitrogen Inc.) were grown in Dulbecco's Modified Eagle's Medium (D-MEM, GIBCO) supplemented with 10% FBS and streptomycin–penicillin (GIBCO). 293FT cells were used within 20 subcultures of the frozen stocks. Monoclonal antibodies against β-actin were obtained from SIGMA. Serum samples from Chilean hantavirus patients were kindly provided by Drs. Hector Galeno and Eugenio Ramirez of the Instituto de Salud Pública de Chile and were decomplemented before use.

2.2. Plasmids

The ANDV glycoprotein precursor coding region (GPC) was isolated from a previously cloned cDNA of isolate CHI-7913 (GeneBank accession number: AY228238 (Tischler et al., 2003), species Andes virus, genus Hantavirus, family *Bunyaviridae* by PCR using specific primers 5-TAGATCTATTATGGAAGGGTGGTATCTGGTTGC-3 forward and 5-AGGACTCGAGGCGGCCGCTTAGACAGTTTTCTTGTGCCC-3 reverse and subsequently cloned into the expression vector pI.18 (kind gift of Dr. Jim Robertson, from the National Institute for Biological Standards and Control, Hertfordshire, UK), giving rise to the plasmid pI.18/GPC. Further, the coding region of ANDV-Gn residues 1-353 was isolated through PCR amplification using primers 5′-CAGAGTCGACAAATGGAAGGGTGGTATCTGGTT-3′ and 5′-CAAGCTCGACTCATACACTGTGATTAGATTCAGG-3′ and cloned into the prokaryotic expression vector pET32(a) (Novagen, Merck Biosciences). Plasmids pGAE1.0 (coding for the green fluorescent protein (GFP) under the Cytomegalovirus (CMV) promoter, flanked by psi sequences and SIV long terminal repeats) and pSIV3+ (coding for SIV Gag-Pol) were previously constructed (Mangeot et al., 2002; Mangeot et al., 2000). pVSV-G (coding for the G protein of VSV), pRRLSIN.cPPT.PGK-GFP.WPRE (coding for GFP under PGK promoter, flanked by psi sequences and Human immunodeficiency virus-1 (HIV-1) long terminal repeats), pCMVΔR8.91 (coding for HIV-1 Gag-Pol), p8.2 (coding for HIV-1 Gag-Pol-Tat-Rev) and PMA243 (coding for HIV-1 envelope proteins) were kindly provided by Dr. Luigi Naldini.

2.3. Development of monoclonal antibodies against ANDV glycoproteins

The monoclonal antibody 2H4/F6 against ANDV-Gc was generated previously in our laboratory (Godoy et al., 2009). To develop monoclonal antibodies against ANDV-Gn, Gn residues 1-353 were synthesized in pET32(a)/Gn(1-353) transformed *Escherichia coli* BL21 cells through a 3 h induction period with 1 mM isopropyl β-D-thiogalagtopyranoside. The thioredoxin 1 (Trx1)-Gn fusion protein was purified from the cell lysates with Ni-nitriloacetate-agarose beads as indicated by the manufacturer (Qiagen). Monoclonal antibodies were generated as previously described (Kohler and Milstein, 1975). The hybridoma cell line 6B9/F5 was identified by ELISA screening in which 6B9/F5 supernatants showed reactivity towards recombinant Gn but not towards the Trx1 protein.

2.4. Lentiviral vector production and purification

293FT cells (3×10^6) grown in 10 cm plates were transfected with three plasmids using the calcium phosphate protocol (Soneoka et al., 1995); Briefly, 8 μg of helper plasmid (pSIV3+, p8.2 or pCMVΔR8.91), 8 μg of plasmid encoding an RNA minigenome (pGAE1.0 or pRRLSIN.cPPT.PGK-GFP.WPRE) and 4 μg of envelope protein coding plasmid (pVSV-G or PMA243) were used. In the case of pseudotyping with ANDV glycoproteins, 8 μg of pI.18/GPC was used. Sixteen hours post-transfection, media was replaced with fresh D-MEM. At 48 h post-transfection, supernatants containing pseudotyped particles were centrifuged at $800 \times g$ for 10 min and filtered through a 0.45 μm PVDF pore-size filter. For concentration of particles, filtered supernatants were ultracentrifuged at $100{,}000 \times g$ for 75 min. Viral pellets were resuspended overnight at 4 °C in RPMI (Mangeot et al., 2002).

2.5. Cell transduction

Serial dilutions of vector-containing suspensions were used to transduce 5×10^4 cells seeded the day before in 12-well plates. Two hours later, medium was replaced by fresh medium. After 70 additional hours, cells were trypsinized, resuspended in PBS/PFA 1% and cell transduction analyzed by detection of GFP expression through flow cytometry (FACScan; Becton Dickinson). Transduction titers were calculated using the percentage of GFP positive cells counting 100,000 cells for each independent experiment ($n \geq 3$).

2.6. Detection of ANDV glycoproteins

To detect cell surface proteins, 293FT cells (3×10^6) grown in 10 cm plates were transfected with 8 μg of pI.18/GPC or the empty pI.18 plasmid as described above. Forty-eight hours post-transfection, cell surface proteins were biotinylated using a cell surface protein isolation kit (Pierce). ANDV glycoproteins and β-actin were detected by Western blot analysis using the biotinylated (plasma membrane proteins) and unbiotinylated (intracellular proteins) fractions. Protein samples were resolved by 12.5% Tris–glycine polyacrylamide gel electrophoresis and transferred onto 0.45 μm pore-size nitrocellulose membrane. Gc and Gn proteins were detected using monoclonal antibodies 2H4/F6 and

6B9/F5 (1:1000), respectively. β-Actin was detected using monoclonal antibody anti-β-actin 1:5000. Primary antibodies were detected with anti-mouse immunoglobulin antibodies conjugated to horseradish peroxidase (Sigma) 1:5000. Detection of peroxidase was performed with a chemiluminescence substrate (SuperSignal® West Pico, Pierce) following manufacturer instructions. To detect glycoproteins in ANDV pseudotyped particles, 20 μl of purified supernatant was resolved by 12.5% Tris–glycine polyacrylamide gel electrophoresis and transferred to a 0.45 μm pore-size nitrocellulose membrane. Gc and Gn proteins were detected with specific monoclonal antibodies as described above.

2.7. Measurement of reverse transcriptase (RT) activity

Lentiviral supernatants were treated with 15 U/mL of benzonase (Novagen) for 15 min at 37 °C (Sastry et al., 2004). Subsequently, benzonase was inactivated through freezing at −80 °C. Next, RT activity of ANDV pseudotyped particles contained in purified supernatants was measured using a RT assay kit (EnzChek®, Invitrogen) under manufacturer instructions. Fluorescence intensity was measured in a microplate reader (Synergy 4 Hybrid Multi-Mode, BioTek) of at least two independent experiments.

2.8. Neutralization of pseudotyped particles

Vector-containing suspensions were incubated with decomplemented convalescence sera from ANDV patients for 1 h at 37 °C. Next, the virus–sera mixture was used to transduce 5×10^4 Vero E6 cells. Two hours later, medium was replaced by fresh medium. After 70 additional hours, cells were trypsinized, resuspended in PBS/PFA 1% and analyzed by flow cytometry (FACScan; Becton Dickinson) as described above.

2.9. Cholesterol depletion assays

Vero E6 cells growing in 12-well plates were washed twice with DMEM and subsequently treated with different concentrations of methyl-β-cyclodextrin (mβCD, Sigma) in DMEM at 37 °C for 30 min. After incubation, the cells were washed twice with DMEM and supernatants containing pseudotyped particles added for 2 h in absence of FBS. Cell viability was determined by trypan blue exclusion. Transduction titers were determined as described above.

3. Results

3.1. Comparison of lentiviral vectors pseudotyped with VSV glycoprotein

Vero E6 cells correspond to the cell line of choice to study hantavirus infection and replication. In order to test which lentiviral vector system gave high transduction efficiencies of Vero E6 cells, HIV-1 and SIV-derived lentiviral vectors were compared. In addition, the HIV-1 based lentiviral vector was produced in presence or absence of the regulatory proteins Tat and Rev. In all experiments, the VSV-G envelope protein was used for lentivector pseudotyping. The HIV-1 lentivector was able to transduce with high efficiency 293T (4.6×10^7 t.u./mL) and HeLa-P4 cells (3.4×10^7 t.u./mL) but with relatively low efficiency Vero E6 cells (5.0×10^6 t.u./mL) (Fig. 1). When the HIV-1 Tat and Rev proteins were present during HIV-1 vector production, a two-fold increase of cell transduction was reached (Fig. 1). With the SIV-derived lentivector, transduction titers decreased about two-fold for 293T (2.18×10^7 t.u./mL) and HeLa-P4 cells (1.61×10^7 t.u./mL) but increased several folds for Vero E6 cells (3.84×10^7 t.u./mL) (Fig. 1). To optimize the efficiency of Vero E6 cell transduction, the SIV lentiviral vector system was selected for pseudotyping with the ANDV-Gn/Gc envelope glycoproteins.

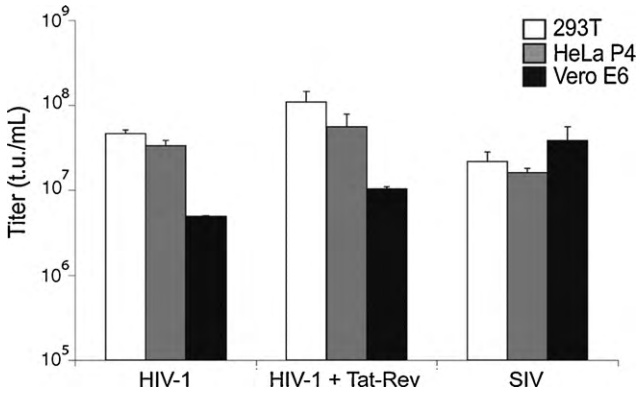

Fig. 1. Transduction efficiencies of lentiviral vectors. The 293T, HeLa-P4 and Vero E6 cell lines were transduced by VSV-G pseudotyped lentiviral vectors, based on HIV-1 with or without the viral Tat and Rev proteins and on SIV. Transduction titers were monitored by FACS analysis counting GFP fluorescent cells among 100,000 cells, for each independent experiment as described in materials and methods.

3.2. Transport of ANDV glycoproteins to the cell surface

One of the characteristic properties of members of the *Bunyaviridae* family consists in the retention of glycoproteins and assembly of virions at the level of the Golgi apparatus (Deyde et al., 2005; Jantti et al., 1997; Kikkert et al., 1999; Salanueva et al., 2003; Shi and Elliott, 2002). However, for hantaviruses it has been shown that at late time points of infection, glycoproteins can reach the plasma membrane (Ogino et al., 2004; Spiropoulou et al., 2003) which led to propose that the plasma membrane of infected cells may resemble an alternative virion assembly site. This notion has been earlier proposed as indicated by electron microscopy studies of cells infected with Sin Nombre virus (Goldsmith et al., 1995). Based on the fact that retroviruses assemble at the plasma membrane (Ivanchenko et al., 2009; Jouvenet et al., 2008; Nguyen and Hildreth, 2000; Satake and Luftig, 1983) and in order to efficiently pseudotype lentiviral vectors with the ANDV glycoproteins, the presence of ANDV-Gn/Gc at the cell surface of transfected cells was examined. To this end, monoclonal antibodies 6B9/F5 anti-Gn and 2H4/F6 anti-Gc were developed (see Section 2 and Godoy et al., 2009, respectively). Surface proteins of ANDV GPC transfected 293FT cells were biotinylated and proteins contained in biotinylated and unbiotinylated fractions were detected by Western blot using the Gn and Gc specific antibodies. Intracellular fractions of GPC transfected cells contained the Gn and Gc proteins with molecular weights corresponding to the native monomeric proteins (70 and 55 kDa, respectively) (Fig. 2). In addition, both proteins were present as higher order multimers, most probably belonging to Gn and Gc oligomers. As expected, ANDV glycoproteins were also present in surface fractions of pI.18/GPC transfected cells (Fig. 2). Absence of membrane damage and subsequent exposure of internal cell membrane proteins during the biotinylation process was verified by the absence of anti-β-actin antibody reactivity. β-Actin was only detected in intracellular but not in surface fractions, confirming that Gn and Gc are located on the cell surface (Fig. 2). When cells were transfected with the empty expression plasmid pI.18, ANDV glycoproteins were not detected (Fig. 2). Altogether, these results show that a substantial proportion of the ANDV glycoproteins are present at the plasma membrane of GPC transfected cells. This further supports the feasibility of pseudotyping lentiviral vectors with ANDV glycoproteins.

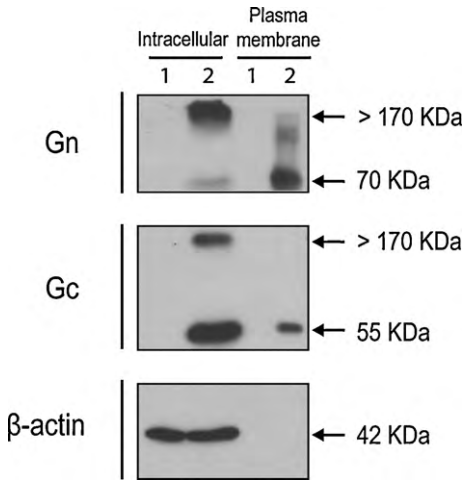

Fig. 2. Cellular distribution of the ANDV glycoproteins. 293FT cells transfected with pI.18 (lane 1) or pI.18/GPC (lane 2) plasmids were surface biotinylated. Subsequently intracellular and surface fractions were separated and subjected to Western immunoblot analysis with anti-ANDV Gn, Gc and β-actin monoclonal antibodies.

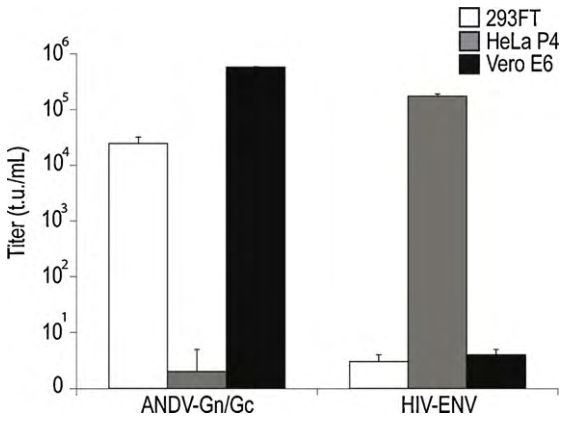

Fig. 3. Transduction of different cell lines with ANDV pseudotyped SIV particles. Transduction titers of lentiviral vectors pseudotyped with the ANDV-Gn/Gc glycoproteins or with the HIV-1 envelope proteins (ENV) were measured in 293FT, Hela-P4 and Vero E6 cells counting 100,000 cells for each experiment.

3.3. Production of ANDV pseudotyped SIV particles

With the aim of pseudotyping SIV vectors with the ANDV glycoproteins, three plasmids coding for ANDV GPC, SIV Gag-Pol and GFP flanked by the SIV long terminal repeats were used for DNA transfection as described in Section 2. Two days after transfection of 293FT cells, supernatants were purified and subsequently used to transduce different cell lines. SIV vectors pseudotyped with ANDV-Gn/Gc were able to transduce Vero E6 cells with titers up to 5.8×10^5 t.u./mL (Fig. 3). The capability of infecting different cells conferred by ANDV envelope proteins was analyzed by measuring the transduction efficiency of other cell lines. Human embryonic kidney derived 293FT cells were transduced with lower efficiency than Vero E6 cells (2.5×10^4 t.u./mL) whilst the ANDV pseudotyped particles were not able to transduce HeLa-P4 cells (Fig. 3). To compare the efficiency of pseudotyping, SIV-derived lentiviral vectors pseudotyped with the envelope proteins of HIV-1 were produced. In this case, HIV pseudotyped particles were highly efficient in transducing HeLa-P4 cells (1.7×10^5 t.u./mL) but not Vero E6 nor 293FT cells, reflecting the absence of adequate entry receptors on these cells (Fig. 3). In summary, these findings indicate that SIV vectors pseudotyped with the ANDV glycoproteins can transduce with high efficiency permissive cell lines and further show that the infection and transduction abilities of the SIV pseudotyped vectors is specific, depending on the characteristics of each cell line.

3.4. Characterization of ANDV pseudotyped SIV particles

To confirm the incorporation of ANDV glycoproteins onto lentiviral vectors, the purified supernatant of the producer cells was analyzed for the presence of ANDV-Gn/Gc glycoproteins and RT activity. ANDV-Gn/Gc was detected by Western immunoblots of supernatants with specific monoclonal antibodies (Fig. 4a). When the empty pI.18 expression plasmid was used to produce lentiviral vectors, neither Gn nor Gc was detected (Fig. 4a) and no vector transducing activity was observed (data not shown). The incorporation of SIV proteins into lentiviral particles was analyzed by measuring the RT activity of supernatants containing pseudotyped particles prepared with VSV, ANDV and HIV envelope proteins. As expected, RT activity was higher in supernatants containing particles pseudotyped with VSV-G, and lower for particles pseudotyped with ANDV-Gn/Gc and HIV envelope proteins (Fig. 4b) consistent with their efficiencies of cell transduction (compare with Fig. 1 and Fig. 3).

3.5. Neutralization of ANDV pseudotyped SIV particle entry

The antigenicity of the lentiviral vectors pseudotyped with ANDV-Gn/Gc was analyzed using sera derived from ANDV infected patients. To monitor neutralization of ANDV pseudotyped particles, they were incubated with patient sera and used to transduce Vero E6 cells. At a 1/50 dilution, different human sera caused a 35–90% reduction of ANDV pseudotyped particle infection (Fig. 5). A dose dependent effect of ANDV pseudotyped particle neutral-

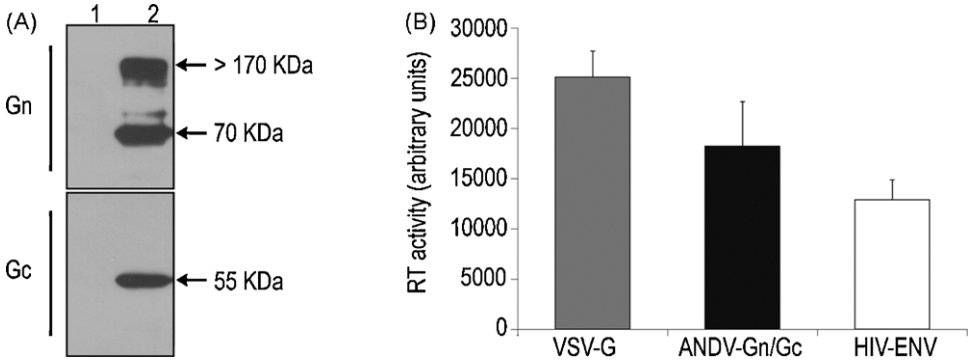

Fig. 4. ANDV glycoproteins incorporation onto lentiviral vectors. (a) Western immunoblot of mock pseudotyped SIV vector (empty pI.18 plasmid, lane 1) or ANDV Gn/Gc pseudotyped (lane 2) using mAbs anti-Gn 6B9/F5 and anti-Gc 2H4/F6. (b) RT activity present in the supernatants of cells producing VSV-G, ANDV-Gn/Gc or HIV-1 ENV pseudotyped lentiviral vectors.

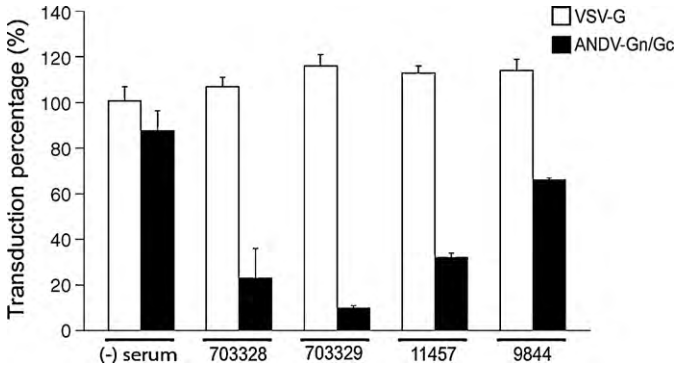

Fig. 5. Neutralization of the infectivity of ANDV pseudotyped SIV particles by sera of ANDV infected patients. Lentiviral vectors pseudotyped with ANDV-Gn/Gc or VSV-G (negative control) were incubated for 1 h with human negative control serum or ANDV patient sera (1/50 dilution) and next used for the transduction of Vero E6 cells. Titers are expressed as a percentage of the transduction titers obtained in absence of human sera (Mock).

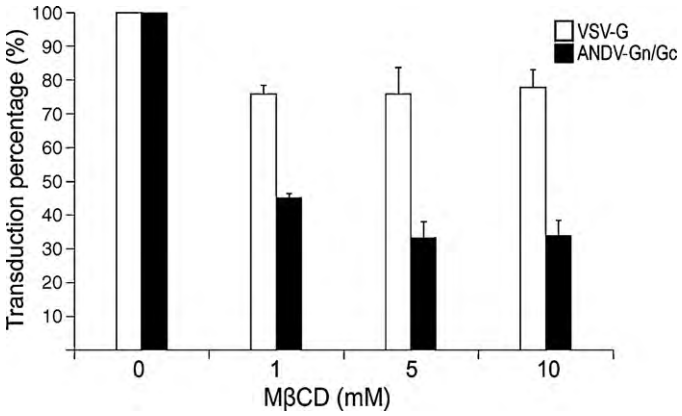

Fig. 6. Effect of cholesterol depletion on pseudotyped particle entry. Vero E6 cells were treated for 30 min with increasing concentrations of mβCD and subsequently infected by lentiviral vectors pseudotyped with the ANDV-Gn/Gc (black columns) or VSV-G (white columns). Titers are expressed as the percentage of transduction titers in absence of mβCD treatment.

ization was observed when sera were added at increasing dilutions (data not shown). When VSV-G pseudotyped lentiviral vectors were treated with the same panel of ANDV patient sera, no inhibition was observed. The use of sera to neutralize ANDV pseudotyped particles coincide with our previously published results, in which these sera diminished ANDV infection (Tischler et al., 2005a). The differences in serum dilutions required to neutralize the native ANDV or ANDV pseudotyped particles may be related to a high amount of non-infectious pseudotyped particles to which neutralizing antibodies may bind, thereby lowering their neutralizing effect. Similar results have been reported for the neutralization of ANDV pseudotyped particles derived from VSV (Ray et al., 2010). Altogether, these results indicate that cell entry of ANDV pseudotyped particles can be inhibited by serum derived from patients infected with the native virus and that the envelope of ANDV pseudotyped particles share antigenic characteristics with ANDV.

3.6. Cholesterol dependency of ANDV pseudotyped SIV particle entry

Cholesterol modulates membrane viscosity and assists in the formation of membrane microdomains termed lipid rafts (Simons and Ikonen, 1997) which act as docking sites for specific proteins involved in many important cell signalling processes (Simons and Toomre, 2000). These include among many others, endocytosis (Hailstones et al., 1998; Heiniger et al., 1976; Rodal et al., 1999; Rothberg et al., 1990) and cell attachment of pathogens (Simons and Ehehalt, 2002). Further, cholesterol is required for the virus-cell fusion process of some viruses (Smit et al., 1999; Waarts et al., 2002). Yet, the role of cholesterol on hantavirus entry has not been determined. With the purpose of characterizing cholesterol dependence of ANDV cell entry, cholesterol was depleted from target cells. To that end, the cyclic oligosaccharide mβCD was used. When Vero E6 cells were treated for 30 min with different concentrations of mβCD before incubation with ANDV pseudotyped particles, transduction of cells was diminished up to 70% in a dose dependent manner (Fig. 6). In contrast, when cholesterol-independent VSV-G pseudotyped particles were added to cholesterol depleted cells, a modest change of cell transduction efficiency was observed which was not dose dependent (Fig. 6). In all cases, no cytopathic effect, which may have been originated by mβCD treatment, was observed as measured by the exclusion of trypan blue (data not shown). The results suggest that ANDV entry depends on the presence of cholesterol in the target cell membrane.

4. Discussion

In the present work lentiviral vectors pseudotyped with ANDV-Gn/Gc were developed to study the early steps of ANDV cell infection. HIV-1 and SIV based lentivectors are currently used for efficient gene delivery and stable genetic transformation of dividing and non-dividing cells (Frecha et al., 2008a; Mangeot et al., 2000; Negre et al., 2000; Trono, 2000). Such lentiviral vectors represent powerful and safe gene transfer tools since they can be easily produced at high titers and the generation of replication-competent retroviruses is very unlikely (Mangeot et al., 2002). HIV-1 derived vectors with or without Tat and Rev proteins can efficiently transduce the human cell lines HeLa-P4 and 293FT, but much less so for Vero E6 cells. In comparison, SIV-derived vectors were more efficient in transducing the monkey derived cell line Vero E6. Such differences in transduction efficiencies of monkey cells are probably related to post-entry restrictions against HIV-1, by innate cellular responses which block the uncoating process of the virion capsid structure (Himathongkham and Luciw, 1996; Hofmann et al., 1999; Stremlau et al., 2004).

The possibility to produce ANDV pseudotyped particles based on lentiviral vectors was indicated by the fact that the ANDV glycoproteins localized, at least in part, at the level of the plasma membrane where lentivirus assembly takes place. In permissive cell lines, transduction efficiencies by ANDV pseudotyped lentiviral vectors were similar to that found for HIV-1 envelope protein pseudotyping, indicating that envelope protein accumulation at the plasma membrane was not a limiting factor for lentiviral vector pseudotyping. The incorporation of ANDV glycoproteins onto the lentiviral particles was investigated by means of the reactivity of ANDV-Gn/Gc specific antibodies developed in this work and in Godoy et al. (2009). The Gc glycoprotein migrated as a unique band while Gn was found in multiple forms, probably representing different oligomeric structures. In agreement with this, a recent work indicates the oligomerization of Gn and Gc of Tula virus (*Hantavirus* genus) virions might be comprised of Gn homotetramers interconnected with Gc homodimers (Hepojoki et al., 2009). This observation may explain why in ANDV pseudotyped particles the ANDV Gn protein was found in higher amounts than the Gc protein. To further characterize the organization of ANDV-Gn/Gc at the surface of lentiviral particles, their reactivity with sera obtained from ANDV infected patients was examined. Since these sera were able to neutralize ANDV pseudotyped particle infection of Vero E6 cells, ANDV-Gn/Gc probably adopt a structural organization similar to that prevailing on ANDV virions.

The highest transduction efficiencies by SIV lentiviral vectors pseudotyped with the ANDV glycoproteins were reached with Vero E6 cells which are typically used for *ex vivo* replication of hantaviruses. 293FT cells were also susceptible to transduction, but efficiencies were 10 times lower than with Vero E6 cells. These findings differ from previous results (Ray et al., 2010) where a two times higher infection efficiency of 293T compared to Vero E6 cells was obtained. One possible explanation may be that 293T cells have a higher metabolism and growth kinetic than Vero E6 cells, thus during the same period of time they may express higher levels of the luciferase reporter gene than the same number of infected Vero E6 cells. The present lentiviral vector system provides the advantage to correlate the real GFP reporter gene expression with the number of GFP expressing cells and thereby allows an unambiguous interpretation of cell infection for a large panel of different cell types.

Using lentiviral vectors pseudotyped with the ANDV glycoproteins, it was found that infection was strongly diminished when cholesterol was depleted from target cell membranes. Although this data should be corroborated with the native ANDV, it suggests that cholesterol is required for ANDV cell entry in a manner similar to that for the Crimean-Congo hemorrhagic fever virus (Simon et al., 2009). In cell membranes, cholesterol is highly enriched in raft domains (Simons and Ikonen, 1997) where many signalling proteins and receptors tend to accumulate (Foster et al., 2003; Simons and Toomre, 2000; von Haller et al., 2001). The local enrichment of molecules in membrane lipid rafts is thought to facilitate mutual interactions (Simons and Toomre, 2000). Cholesterol depletion is thought to produce raft disintegration (Smart and Anderson, 2002) thus changing protein distribution in membranes. In this context our results may be explained in two different ways: (i) cholesterol depletion may interfer with virus uptake via endocytosis or (ii) cholesterol depletion may have affected receptor distribution and thereby impaired cellular signalling for viral uptake through endocytosis, a process occurring through receptors clustering upon virus binding (Goldstein et al., 1979; Marsh and Helenius, 2006; Metzger, 1992). Studies to determine whether hantaviruses directly associate with lipid rafts are in progress.

Lentiviral vectors are becoming widely used for gene delivery for research purposes and biomedical applications (Cockrell and Kafri, 2007; Dissen et al., 2009; Frecha et al., 2008b; Naldini, 2009). Furthermore, lentiviral vectors can be pseudotyped using distinct viral envelope proteins in an attempt to restrict gene delivery to specific cells *in vivo* (Cronin et al., 2005). In this context, ANDV pseudotyped particles could be used for *in vivo* assays to identify cells and tissues infected in living organisms. At the same time, ANDV pseudotyped lentiviral particles should permit to test *in vivo* the effect of cell entry inhibitors. In conclusion, ANDV pseudotyped lentiviral vectors offer a safe, rapid and quantitative tool to study ANDV glycoprotein functions, virus-cell entry and inhibition in cell cultures and animal models.

Acknowledgements

The authors thank Dr. Pablo Valenzuela from Fundación Ciencia para la Vida for supporting and advising these studies. Drs. Eugenio Ramirez and Hector Galeno from the Instituto de Salud Pública de Chile for providing sera samples from Chilean hantavirus patients.

This work was financed by grants FONDECYT 1100756, USS 5049, CONICYT PFB-16 and UNAB DI-05-08/I. NCM is supported by Universidad Andrés Bello doctoral fellowship MECESUP UAB0602. JLD is supported by INSERM and FINOVI funding.

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