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Role of heparan sulfate in entry and exit of Ross River virus glycoprotein-pseudotyped retroviral vectors

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ABSTRACT

Variants of Ross River virus (RRV) that bind to heparan sulfate (HS) were previously selected by serial passaging in cell culture. To explore the effects of mutations that convey HS utilization, we pseudotyped Moloney murine leukemia virus (MoMLV), with the RRV envelope. We substituted amino-acid residues 216 and 218 on RRV-E2-envelope glycoprotein with basic amino-acid residues, because these mutations confer affinity for HS upon RRV. However, T216R-RRV- and N218R-RRV-pseudotyped viruses possessed lower transduction titers, and we demonstrated that HS-affinity impeded release of pseudotyped virus from producer cells. Addition of heparinase to HS-expressing target cells reduces the transduction efficiency of the T216R-RRV- and N218R-RRV-pseudotyped viruses, whereas no such effect is seen in cells lacking HS. Under appropriate conditions, these T216R-RRV- and N218R-RRV-pseudotyped viruses have enhanced capacities for transducing HS-expressing cells. General principles concerning viral adaptation to the use of attachment factors and design of pseudotyped viral vectors are discussed.

1. Introduction

Viruses are commonly used as vectors to deliver a desired gene to the target cells for the purpose of gene therapy or gene transfer. Retroviruses, such as human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), and Moloney murine leukemia virus (MoMLV) may be useful as gene-delivery vectors, because they allow stable transfer of genes under the influence of strong promoters to cells (Naldini et al., 1996; Kafri et al., 1997; Poeschla et al., 1998; Noh et al., 2010). Whereas the recognition and infection of host cells by viruses depends on their envelope proteins, incorporating the envelope proteins from other viruses can convey to the parent virus the ability to enter different cells than those it normally infects. These chimeric viruses are called pseudotyped viruses. Recombinant retroviruses and lentiviruses bearing alphavirus glycoproteins have been created and have been shown to be efficient gene transfer/therapy vectors (Sharkey et al., 2001; Kang et al., 2002). Apart from their utility as gene transfer/therapy vectors, alphavirus pseudotypes have been used as tools to study the entry of alphaviruses.

Alphaviruses are arthropod-borne viruses that cause severe infections in a wide range of vertebrates. The clinical manifestations in infected humans include fever, rash, arthralgia, myalgia, headaches and polyarthritis (Strauss and Strauss, 1994). Alphaviruses are classified into two categories: (i) Old World viruses, including Ross River virus (RRV), Semliki Forest virus (SFV), Chikungunya virus (CHIKV) and Sindbis virus (SINV) and (ii) New World viruses, Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus (EEEV) (Strauss and Strauss, 1994).

An alphavirus is an enveloped, single-stranded, positive-sense RNA virus (11.5 kb) (Strauss and Strauss, 1994). The RNA genome is surrounded by the capsid shell, which is made up of 240 units of the capsid protein. This in turn is surrounded by an envelope consisting of the glycoproteins E1, E2, and E3 embedded in a cell-membrane-derived lipid bilayer. Each spike is a trimer of E1/E2 heterodimers (Cheng et al., 1995), and E3 is non-stoichiometrically abundant. The envelope and the inner nucleocapsid core share icosahedral symmetries with triangulation number T = 4 (Strauss and Strauss, 1994; Cheng et al., 1995).

RRV, like other alphaviruses, has a wide host range and tissue tropism, as their infectious cycle requires infecting both mammals and insects (Kielian et al., 2010; Jose et al., 2009; Kahl et al., 2004). Alphaviruses enter the cells via receptor-mediated endocytosis (Sharkey et al., 2001; Kielian et al., 2010; DeTulleo and Kirchhausen, 1998). Binding to receptors and attachment factors is mediated by E2 glycoproteins, whereas E1 glycoproteins mediate fusion of the viral and endosomal membranes (Strauss and Strauss, 1994; Smith et al., 1995). Many proteinaceous receptors are thought to be responsible for internalization of alphaviruses particles. However, these receptors have not been characterized completely (Kielian et al., 2010; Jose et al., 2009).

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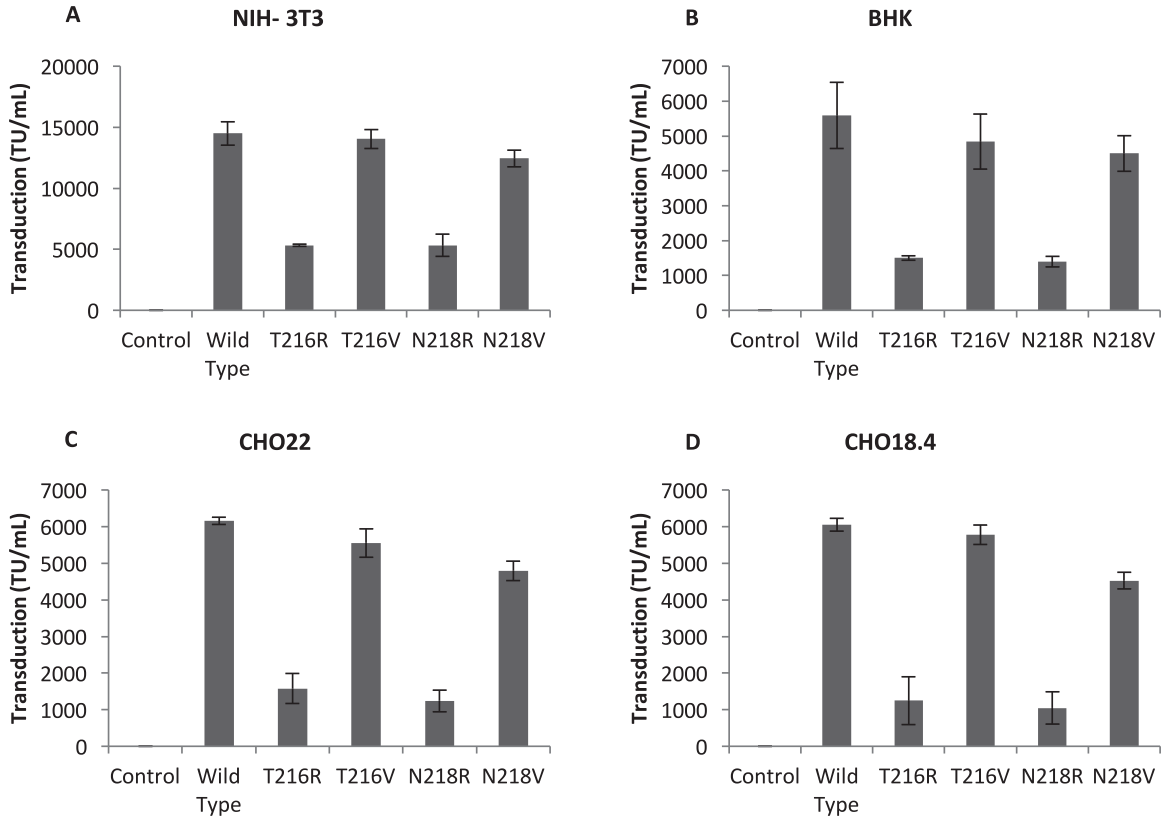

Fig. 1. T216R-RRV and N218R-RRV pseudotyped viruses have lower transduction titres. Supernatant media overlying producer ØNXnls*lac*Z cells producing WT-RRV, T216R-RRV, T216V-RRV, N218R-RRV and N218V-RRV pseudotyped viruses respectively were collected and passed through a 0.45 μm filter. Hexadimethrine bromide (5 μg/mL) was added to the filtered supernatant media. This media was used to transduce target cell-lines (A) NIH 3T3, (B) BHK, (C) CHO22 (parent cell-line) and (D) CHO18.4 (cell-line not expressing HS). The transduced target cells were stained with X-gal and transduction titers were calculated. The experiments were repeated thrice. The values shown by the graph represent the mean TU/mL values (± SD) calculated from these experiments.

Cell-surface attachment factors, which facilitate the initial binding of alphaviruses and thus increase the concentration of virus particles on the cell surface, such as DC-SIGN and L-SIGN have been suggested to play a role in alphaviral entry (Klimstra et al., 2003). Heparan sulfate (HS), an attachment factor used by a number of viruses, such as human papilloma virus, hepatitis C virus, most herpes viruses, adeno-associated virus, dengue virus, vaccinia virus, yellow fever virus and human respiratory syncytial virus (Giroglou et al., 2001; Barth et al., 2003; Shukla and Spear, 2001; Summerford and Samulski, 1998; Chen et al., 1997; Chung et al., 1998; Germi et al., 2002; Feldman et al., 2000), has also been proposed to be involved in alphaviral entry (Byrnes and Griffin, 1998; Zhu et al., 2010; Gardner et al., 2013). In particular, it has been proposed that capacity to use HS as an attachment factor plays a role in the tissue tropism of Eastern equine encephalitis virus (Gardner et al., 2011).

HS is ubiquitously found in the human body on cell surfaces and in the extracellular matrix. It is a glycosaminoglycan made up of repeating units of uronic acid and glucosamine with varying degrees of sulfation, which confers a negative charge (Lopes et al., 2006). This negative charge allows ionic interaction with cationic molecules. SINV has been shown to use HS as an attachment factor (Byrnes and Griffin, 1998). It was later shown that serial passaging in cell culture led to adaptive mutations in SINV, which led to its selection of HS as an attachment factor (Klimstra et al., 1998). Similar studies done in RRV revealed that serial passaging in chick embryo fibroblasts led to mutations at position 218 on the E2 envelope glycoprotein that resulted in replacement of the original asparagine residue with lysine (Kerr et al., 1993). Incorporation of these N218R and N218K mutations in RRV was shown to permit RRV to utilize HS as an attachment factor (Heil et al., 2001). It was also shown that even though substitutions did not result in formation of the HS-binding motifs XBBXBX or XBXBBBX, the electrostatic interaction between negatively charged HS and basic amino acids residues clustered around the receptor binding region of E2 glycoprotein, made utilization of HS as an attachment factor possible (Heil et al., 2001; Zhang et al., 2005).

We have previously described pseudotyping of recombinant retroviruses and lentiviruses with alphaviral glycoproteins, including those of RRV (Sharkey et al., 2001; Kang et al., 2002; Kahl et al., 2004). In vivo gene transfer to hepatocytes and neuroglial cells was successfully achieved by pseudotyping lentivirus with RRV envelope glycoproteins (Kang et al., 2002). In order to further explore potential advantages of alterations to the viral glycoproteins to transduction by RRV-MoMLV pseudotyped virus, we created RRV-envelope glycoproteins with the substitutions of basic amino-acid residues (T216R and N218R). Residue T216 has been shown to be involved in receptor binding (Smith et al., 1995; Vrati et al., 1988). Previously data had been provided that NI218R-RRV utilized HS as an attachment factor for entry, but no data had been published concerning T216R-RRV (Heil et al., 2001). In addition, in the context of the alphavirus virion there are protein-protein interactions between the capsid and E2 glycoproteins (Aggarwal et al., 2012; Tang et al., 2011; Jose et al., 2012; Wilkinson et al., 2005; Brown et al., 2018). It is possible that they affect the conformation of the glycoprotein and thereby its capacity to bind to HS. It is known that the conformation of the cytoplasmic domains of viral glycoproteins can affect their function in membrane fusion and viral entry (Bagai and Lamb, 1996; Mulligan et al., 1992; Sergel and Morrison, 1995; Taylor and Sanders, 2003; Ragheb and Anderson, 1993; Silverman et al., 2012). Therefore, it was desirable to examine whether use of HS as an attachment factor by mutated RRV glycoproteins is independent of the interaction between the E2 glycoprotein and capsid. We demonstrate

that these substitutions promote the utilization of HS by RRV glycoprotein-pseudotyped retrovirus. Interestingly, these substitutions also appear to reduce the release of pseudotyped virus from producer cells. Incorporation of substitutions therefore has both advantages and disadvantages for gene transduction by alphavirus-pseudotyped retroviruses and lentiviruses.

2. Results

2.1. Reduced transduction by T216R-RRV and N218R-RRV pseudotyped viruses is of both HS expressing as well as nonexpressing cell lines

Substitution of arginine for asparagine-218 (N218R) of RRV-E2 glycoprotein resulted in increased replication of RRV in BHK cells (Heil et al., 2001). To determine whether substituting amino-acid residues threonine-216 or aspargine-218 of RRV-E2 glycoproteins with basic amino-acid residues conveyed the utilization of HS upon pseudotyped retroviruses and thus increased their transduction efficiency, transduction assays were performed using these T216R-RRV and N218R-RRV pseudotyped viruses. Pseudotypes with no substitutions in envelope glycoproteins, WT-RRV, and pseudotypes with envelope glycoproteins containing substitutions of residues 216 or 218 with valine, T216V-RRV and N218V-RRV, were used as controls. The data, however, show that the transduction titers of WT-RRV, T216V-RRV, N218V-RRV pseudotyped viruses were higher than those of pseudotyped viruses with basic amino-acid substitutions (T216R-RRV and N218R-RRV) in both HS expressing as well as nonexpressing cell lines (Fig. 1).

2.2. Reduced incorporation of T216R-RRV and N218R-RRV envelope glycoproteins into pseudotyped virus

The lowered transduction titers observed with the T216R-RRV and N218R-RRV pseudotyped viruses would appear to be in conflict with the results previously obtained with the mutant RRV viruses that displayed increased replication. To further investigate the reason for lower transduction titers in T216R-RRV and N218R-RRV, immunoblot assays were carried out. Immunoblot analysis (Fig. 2) of the pellets containing the viral particles showed that there was very little incorporation of the T216R-RRV and N216R-RRV-envelope glycoproteins as compared to that of the WT-RRV, T216V-RRV and N218V-RRV-envelope glycoproteins. The amount of capsid protein in the viral pellets observed however was equal across all the samples. Thus, the virus particles from T216R-RRV and N218R-RRV samples were lacking the envelope around the retroviral cores. This could explain the reason for lower transduction titers in these samples. Further, the amounts of envelope glycoproteins and capsid proteins in the lysates obtained from producer ØNXnls*lac*Z cells were the same across in all samples. This shows that the mutations did not affect the production of the T216R-RRV and N218R-RRV envelope glycoproteins. The cell debris, which is the insoluble part of the producer cell (parts of the cells resistant to solubilization by 1% Triton-X 100 lysis solution), however, contained higher levels of the glycoproteins in the T216R-RRV and N218R-RRV expressing cells as compared to wild-type, T216V-RRV and N218V-RRV expressing cells. The quantities of the capsid proteins were the same across all the samples. This suggests that the envelope glycoproteins of T216R-RRV, N218R-RRV are being retained within the producer cells and predominantly naked MoMLV cores lacking the envelope were released.

2.3. Attachment of T216R-RRV and N218R-RRV envelope glycoproteins to HS leads to retention of these glycoproteins within the cells producing them

Immunoblot assays showed that the envelope glycoproteins of T216R-RRV and N218R-RRV were retained within the producer ØNXnlslacZ cells. To study the role of HS in the retention of the envelope glycoproteins, the glycoprotein-expressing plasmids were transfected into CHO22 (HS-expressing) and CHO18.4 (HS-nonexpressing) cell-lines. An immunofluorescence assay demonstrated that there was excess retention of T216R-RRV and N218R-RRV envelope proteins in CHO22 cells as compared to WT-RRV, T216V-RRV and N218V-RRV envelope glycoproteins (Fig. 3A and Supplemental Fig. 2A). In CHO 18.4, a cell line that does not express HS, no excess retention of T216R-RRV and N218R-RRV envelope glycoproteins was seen in comparison to cells expressing the WT-RRV, T216V-RRV and N218V-RRV envelope glycoproteins (Fig. 3B and Supplemental Fig 2B). It can be concluded that the affinity of T216R-RRV and N218R-RRV glycoproteins towards HS leads to their intracellular retention in virus-producing cells, and predominantly naked MoMLV cores, incapable of transducing target cells, are released from cells producing these mutant envelope glycoproteins.

2.4. Attachment of T216R-RRV and N218R-RRV envelope glycoproteins to HS hinders the release of virus particles

To determine whether the affinity of RRV envelope glycoproteins towards HS expressed on the surface of the producer cells affects the release of virus particles, Heparinase I was added to the producer ØNXnls*lac*Z cells. Heparinase I cleaves HS from the cell surface by breaking the α (Naldini et al., 1996; Kafri et al., 1997; Poeschla et al., 1998; Noh et al., 2010) glycosidic bond of HS. The aim of this

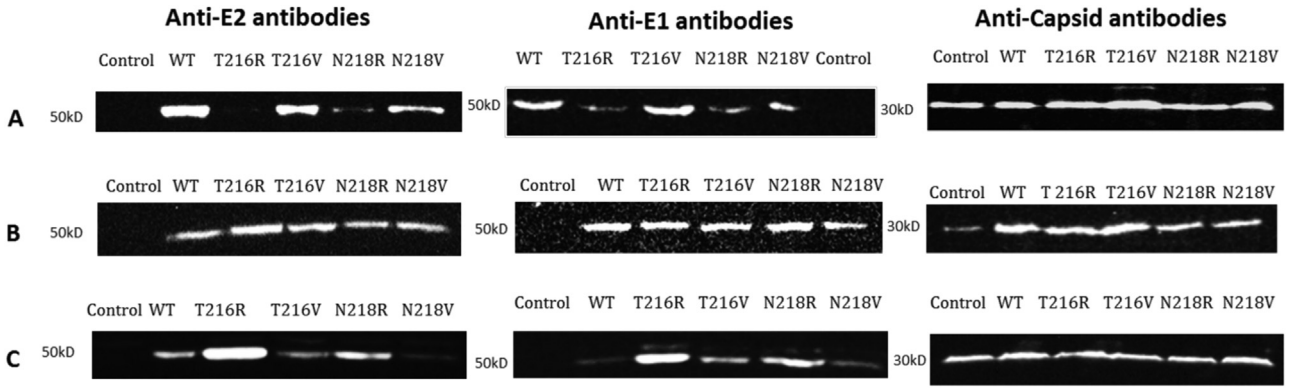

Fig. 2. T216R-RRV and N218R-RRV envelope glycoproteins are inefficiently incorporated in pseudotyped virus. Virus pellets (A) were spun down from supernatant media overlying producer ØNXnls*lac*Z cells transfected with respective RRV-envelope encoding plasmids, while the lysate (B) and the cell debris (C) were obtained by lysing the same producer ØNXnls*lac*Z cells. These preparations were run on SDS-PAGE gels and transferred on to nitrocellulose paper. RRV-envelope glycoproteins (E1 and E2) and MoMLV Capsid proteins in these samples were detected by immunoblotting with rabbit anti-E1, rabbit anti-E2 and goat anti-Capsid (p30) polyclonal primary antibodies (1:5000) respectively, followed by secondary antibodies coupled with horseradish peroxidase. These experiments were performed thrice.

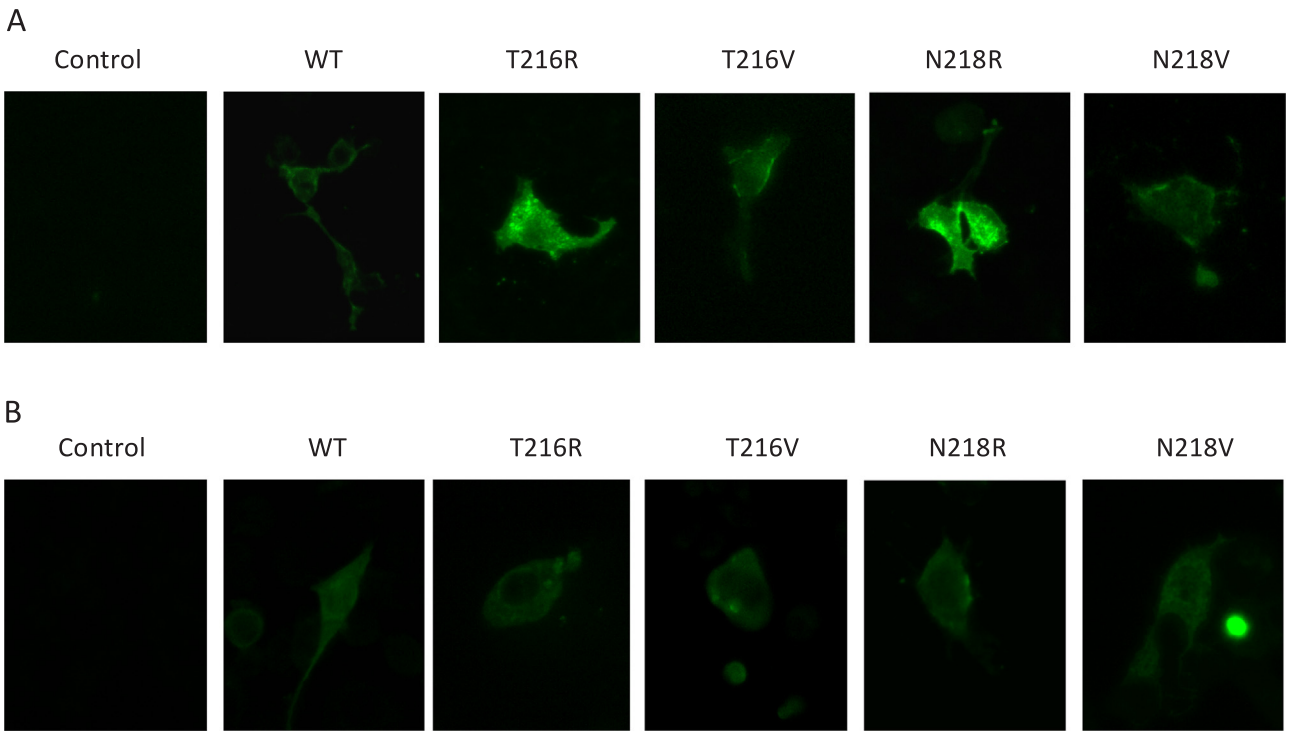

Fig. 3. Intracellular retention of T216R-RRV and N218R-RRV in HS expressing cells. CHO22 cells (A) and CHO18.4 (B) were transfected with respective RRV-envelope plasmids. After fixing and permeabilizing the cells, they were incubated with primary Anti-E2 (rabbit) antibodies. After washing off the excess primary antibodies, cells were incubated with Alexa Fluor 488-coupled anti-rabbit antibodies (green fluorescence) and visualized under fluorescent microscope.

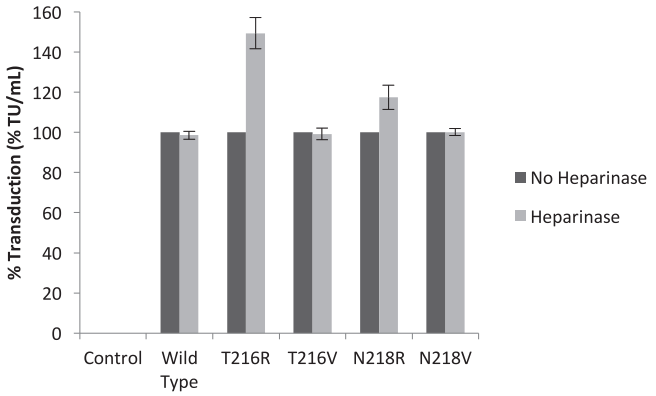

Fig. 4. Addition of Heparinase I to Producer ØNXnlslacZ cells releases T216R-RRV and N218R-RRV pseudotyped viruses. Producer ØNXnlslacZ cells transfected with respective RRV-envelope plasmids are treated with Heparinase I. Supernatant media were collected from these cells and used for transduction of target cells (BHK) as described earlier. Transduction titers were calculated for each experiment which was repeated thrice. The graph represents transduction titers, where 100% TU/mL for WT, T216R, T216V, N218R and N218V are 6090, 1650, 3860, 1760 and 4510 TU/mL respectively. The effect of addition of Heparinase I to producer cells is shown statistically using the paired *t*-Test in the Supplemental Table 1.

experiment was to see whether cleaving of HS from the surface of these producer cells facilitates the release of T216R-RRV and N218R-RRV pseudotyped viruses and thus increases their transduction efficiency. T216R-RRV and N218R-RRV pseudotyped viruses collected from producer ØNXnls*lac*Z cells treated with Heparinase I show a small but statistically significant increase in transduction titers as compared to viruses collected from cells not treated with Heparinase I (Fig. 4 and Supplemental Table 1). Addition of Heparinase I to producer ØNXnls*lac*Z cells producing WT-RRV, T216V-RRV and N218V-RRV pseudotyped viruses shows no effect on their respective transduction efficiency. Thus, HS expressed on the surface of producer ØNXnls*lac*Z cells hinders the process of release of T216R-RRV and N218R-RRV pseudotyped viruses. The small effect observed reflects the fact that the observed reduction of the transduction titer of the T216R-RRV and N218R-RRV pseudotyped viruses in HS-expressing cells results predominantly from intracellular retention of the glycoproteins rather than extracellular retention of glycoprotein-bearing virus particles.

2.5. T216R-RRV and N218R-RRV pseudotyped viruses utilize HS as an attachment factor

To investigate whether T216R-RRV and N218R-RRV pseudotyped viruses utilize HS as an attachment factor, Heparinase I was added to the target cells. The data demonstrate (Fig. 5 and Supplemental Tables 2, 3 and 4) that in BHK and CHO22 cells, as the concentration of Heparinase I increases, the transduction titers of T216R-RRV and N218R-RRV pseudotyped viruses decrease, whereas transduction titers of WT-RRV, T216V-RRV and T218V-RRV pseudotyped viruses do not change drastically. However, in CHO18.4, a cell-line not expressing HS, the transduction titers remain equal across all samples irrespective of Heparinase I concentration. In addition, whereas transduction by WT-RRV, T216V-RRV and T218V-RRV pseudotyped viruses in CHO22 and CHO18.4 cells is approximately equivalent, transduction by T216R-RRV and N218R-RRV pseudotyped viruses is higher in CHO22 cells than in CHO18.4 cells. Furthermore, treatment of the CHO22 cells with heparinase reduces transduction by the T216R-RRV and N218R-RRV pseudotyped viruses approximately to the level seen in CHO18.4 cells. Therefore, in cell lines expressing HS, the transduction efficiency of T216R-RRV and N218R-RRV pseudotyped viruses decreases, as the amount of cell surface HS decreases, whereas no such effect is seen in cell lines not expressing HS. Thus, these data demonstrate that T216R-RRV and N218R-RRV pseudotyped viruses utilize HS as an additional attachment factor.

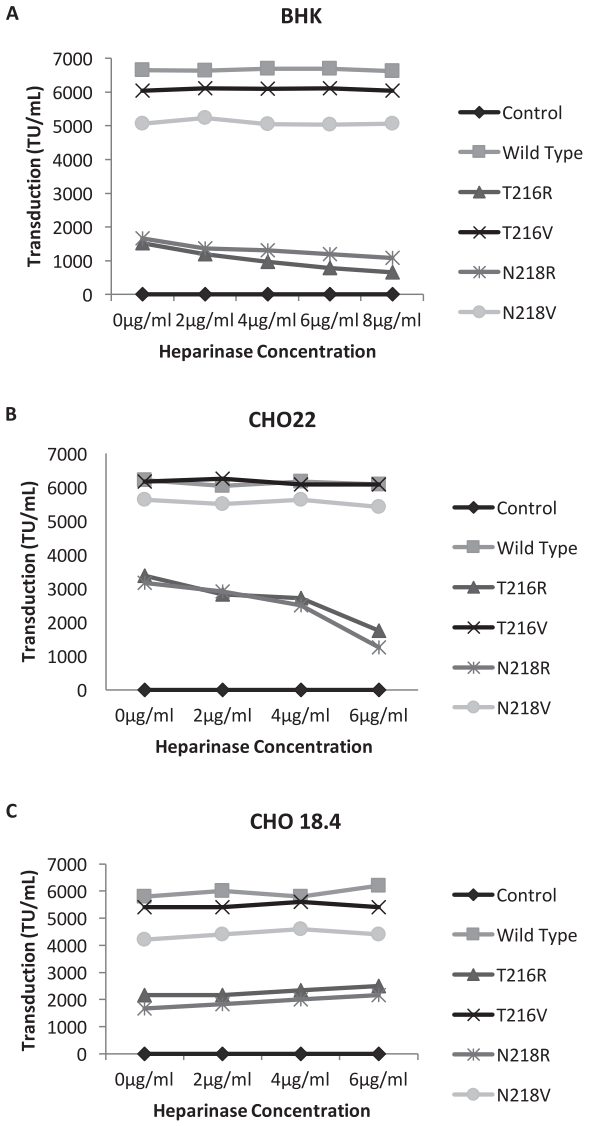

Fig. 5. Heparinase treatment of target cells reduces transduction by T216R-RRV and N218R-RRV pseudotyped viruses. Target cells (A) BHK, (B) CHO22 and (C) CHO18.4 were treated with increasing concentrations of Heparinase I for 1 h before transducing them with WT-RRV and mutant pseudotyped virus, respectively. The experiments were performed in triplicate. The correlation between concentrations of Heparinase I and transduction titers for each sample was determined by calculating Pearson coefficients using Minitab software in (Supplemental Table 2) BHK, (Supplemental Table 3) CHO22 and (Supplemental Table 4) CHO18.4 cell lines.

2.6. Transduction efficiency of T216R-RRV and N218R-RRV is higher in cell lines expressing HS

Intracellular retention of the T216R-RRV and N218R-RRV glycoproteins results in markedly reduced transduction efficiencies by the pseudotyped virus recovered from the supernatant medium of producer cells. To investigate whether attachment to HS expressed on target cells increases the transduction efficiency by the virus particles bearing the low quantities of T216R-RRV and N218R-RRV glycoproteins that are released, normalization of virus concentration by glycoprotein content was performed. The RRV-E2 envelope glycoprotein content of the virus particles collected by ultracentrifugation of the supernatant media of each of the pseudotyped-virus producing cells was measured by immunoblotting. The data show (Fig. 6) that after normalization based on the amount of RRV-E2 glycoprotein, the transduction efficiency for T216R-RRV and N218R-RRV pseudotyped viruses was significantly higher than that of wild type, T216V-RRV and T218V-RRV pseudotyped viruses in NIH 3T3, BHK and CHO22 cells. In CHO18.4, however, there was no significant difference in the transduction efficiency across these samples. In another analysis, we also transduced NIH-3T3 cells with an equal number of wild-type or mutant RRV glycoprotein-bearing virus particles and then normalized the results based upon the RRV-E2 glycoprotein content present in the media used for transducing the cells (Supplemental Fig.1). Together these results support the conclusion that in cell lines expressing HS, when the amount of RRV-E2 glycoprotein protein is equal across all the samples, T216R-RRV and N218R-RRV pseudotyped viruses have higher transduction titers than wild-type, T216V-RRV and N218V-RRV pseudotyped viruses. It appears probable that if there were equal numbers of wild-type and mutant glycoproteins per virion then the T216R-RRV and N218R-RRV pseudotyped viruses would have higher transduction titers on HS-expressing cells.

3. Discussion

Pseudotyped viruses have many experimental and clinical applications. Gene therapy/transfer is one of the important applications of these pseudotyped viruses. Based on the therapeutic need, the range of cells targeted by the viral vector for gene therapy/transfer can be either expanded or narrowed by pseudotyping the virus with the envelope of another virus (Sanders, 2002). For the treatment of systemic genetic disorders a viral vector needs to be pseudotyped with an envelope of a virus that has a broad tissue tropism. Since RRV, an alphavirus, has wide host range and tissue tropism, it was incorporated in ØNX pseudotyped system to produce RRV-MoMLV pseudotyped virus (Sharkey et al., 2001). Previous studies showed that pseudotyping with RRV was far less cytotoxic but equally efficient as VSV-G (Kang et al., 2002). Retrovirus pseudotyped with RRV envelope glycoproteins was successfully used for in vivo gene transfer in hepatocytes, glial cells, muscle tissue and airway epithelial tissue (Kang et al., 2002). To further enhance the transduction efficiency and further broaden the range of cells that can be targeted by the pseudotyped viral vector additional modifications of these envelope glycoproteins are needed.

Earlier studies in RRV show that amino acid T216 on RRV-E2 glycoprotein is the site involved in interaction with the receptors (Smith et al., 1995; Vrati et al., 1988). The RRV48 strain of RRV normally does not utilize HS as an attachment factor. Serial passaging of this strain led to mutations on the E2 envelope glycoprotein in which the original amino-acid residues were replaced with basic amino-acid residues (Kerr et al., 1993). Further studies showed that site-directed mutagenesis involving replacement of asparagine residue at 218 with a basic amino-acid residue confers upon the virus an affinity towards a negatively charged molecule like HS (Heil et al., 2001). Cryo-electron microscopy and image reconstruction studies of RRV-E2 glycoprotein bound to heparin further confirmed this (Zhang et al., 2005). We provide the first published data demonstrating that an arginine substituting for threonine-216 has nearly identical effects to those of the N218R substitution. Our experiments also demonstrate that utilization of HS as an attachment factor by the mutant RRV glycoproteins is independent of RRV glycoprotein-RRV capsid interactions (Aggarwal et al., 2012; Tang et al., 2011; Jose et al., 2012; Wilkinson et al., 2005; Brown et al., 2018).

Substituting the amino-acid residues at 216 and 218 sites on RRV-E2 glycoprotein with basic amino-acid residues in the RRV-MoMLV pseudotyped virus, allows the pseudotyped virus to use HS as an attachment factor. Using HS, an ubiquitous molecule, as an attachment factor increases the concentration of the virus on the cell surface, which can later be internalized by cell surface receptors via endocytosis. Thus, the three-dimensional search for the receptor is reduced to a two-dimensional search, which increases the efficiency of cell entry, thereby enhancing the transduction efficiency of the pseudotyped vector. It has

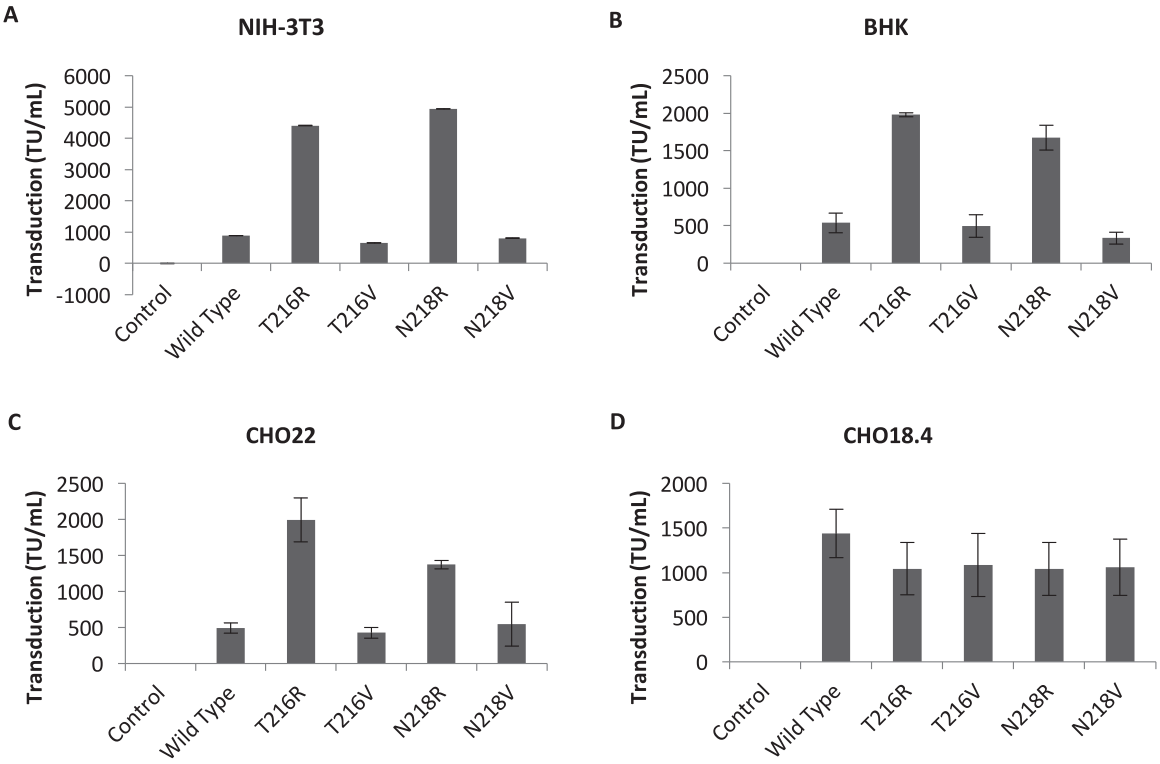

Fig. 6. T216R-RRV and N218R-RRV pseudotyped viruses possess higher transduction efficiencies for HS- expressing target cells when transduction by viruses is normalized by glycoprotein content. Virus was collected by ultracentrifugation from supernatant media overlying producer ØNXnls*lac*Z cells transfected with respective RRV-envelope encoding plasmids. The virus-containing samples were analyzed by SDS-PAGE and transferred onto nitrocellulose paper. RRV-E2 glycoprotein was detected by immunoblotting with rabbit anti-E2 primary and anti-rabbit secondary antibodies coupled with horseradish peroxidase. Using ImageJ software, the amount of RRV-E2 glycoprotein in each virus sample was quantified semiquantitatively, with the mean of imageJ values being 0, 133(± 31.04), 7(± 1.73), 132.6(± 6.42), 14(± 8.18) and 100(± 10.44) for Control, Wild Type, T216R, T216V, N218R and N218V respectively. The amount of media added to target cells (A) NIH-3T3, (B) BHK, (C) CHO22 and (D) CHO18.4, for transduction was normalized against the amount of RRV-E2 glycoprotein present in the given sample. The experiments were conducted at least twice. The values shown by the graph represent the mean TU/mL values (± SD) calculated from these experiments.

been demonstrated that the tissue tropism of North American equine encephalitis virus, an alphavirus, is dependent upon the utilization of HS as an attachment factor (Gardner et al., 2011). Replication in the brain and consequent neurovirulence are increased by the capacity of the NA-EEEV E2 to bind to HS, whereas lymphoid-tissue replication is reduced (Gardner et al., 2011). These data suggest that the tissue specificity of the *in vivo* delivery of genetic material to human subjects may be usefully altered by the capacity of a pseudotyped vector to utilize HS as an attachment factor (Kang et al., 2002; Gardner et al., 2011).

Our data indicate that the capacity of a glycoprotein to bind to HS affects the assembly and release process of pseudotyped virus particles. The envelope glycoproteins with basic amino-acid mutations are not efficiently incorporated into the budding pseudotyped virus. The retention of T216R and N218R RRV-E2 glycoproteins in producer cells (Fig.2 and Fig.3) probably results from the binding of these RRV-E2 glycoproteins to endogenous proteins modified with HS, because HS-based glycosylation of other proteins takes place along the secretory pathway (Banfield, 2011). Secondly, even if the glycoproteins get incorporated into budding pseudotyped virus, the data indicate that their affinity towards HS prevents the release of virus particles from the cell surface. Studies done in SINV have shown that virus particles utilizing HS had reduced viremia resulting in lower pathogenicity. This led to the reversion of virus particles adapted to utilize HS to the wild-type form that had reduced affinity towards HS. One reason proposed for the reduced viremia seen in SINV utilizing HS was decreased dissemination of the virus (Byrnes and Griffin, 2000). Similar studies in VEEV and CHIKV show attenuation of HS-binding mutant viruses due to rapid clearance and decrease in the spread of virus particles (Bernard et al., 2000; Gardner et al., 2014).

This interaction between the HS-utilizing viruses and HS is reminiscent of the interaction between influenza virus and its receptor, sialic acid on target cells (Wiley and Skehel, 2000; Wagner et al., 2002). However, the same attachment factor, when expressed on the cells producing the virus particles, hinders the release of the viral particles from the cell surface (Wagner et al., 2002). The release of influenza virus from cell surfaces is facilitated by the Neuraminidase protein intrinsic to the virus, which cleaves the sialic acid (Air and Laver, 1989; Bucher and Palese, 1975). Because such an intrinsic process for facilitating the release of virus particles is not present in our pseudotyped viral vector, one of our future goals is to develop a producer cell line that does not express HS for the production of this pseudotyped viral vector that utilizes HS as an attachment factor. A general principle arising from these studies is that altering producer cell membrane components may improve pseudotyped virus production through enhancing viral particle release.

The attachment of viruses to cell-surface HS has been proposed both to be an inherent component of viral entry *in vivo* and an adaptation to propagation in cell culture. An important consequence of our studies is the conclusion that utilization of HS as an attachment factor can have deleterious effects on viral release either through intracellular retention of the HS-binding viral proteins or adsorption of the viral particles to producer cells. Herpes simplex virus-1 (HSV-1] utilizes HS as an attachment factor for infection of host cells (Shukla and Spear, 2001; Shukla et al., 1999). Recently it has been demonstrated that increased host heparinase expression is a consequence of HSV-1 infection and that augmentation of heparinase expression correlates with enhanced HSV-1 release (Hadigal et al., 2015). These data reinforce our conclusion that if a virus utilizes an ubiquitous and abundant cell component for entry

then there must be a mechanism for release of the virus from an infected cell if efficient infection is to occur. This issue is likely to be particularly critical when the utilization of the attachment factor appears to arise from an adaptation to growth in cell culture.

4. Material and method

4.1. Cell lines, cell culture and plasmids

Producer ØNXnls*lac*Z cells (a second generation 293-T based retroviral packaging cell line that is stably transfected with a MoMLV *gag-pol* plasmid and MFG.S-nlslacZ plasmid, which produces envelope-protein-deficient, replication-incompetent MoMLV particles that encode nuclear-localized β-galactosidase) (Sharkey et al., 2001); BHK; CHO22; CHO18.4 (a mutant cell line derived from CHO22 not expressing HS) (Heil et al., 2001; Jan et al., 1999) [generously donated by Dr. Richard Kuhn's laboratory] were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and penicillin (10 U/mL) at 37 °C in 5% CO_2. NIH 3T3 cells were grown in DMEM with 10% calf serum (CS) and penicillin (10 U/mL) at 37 °C in 5% CO_2. Regions of the RRV cDNA containing amino-acid substitutions T216R, T216V, N218R or N218V (Heil et al., 2001) [kind gifts from Dr. Richard Kuhn's laboratory] were removed through restriction endonuclease cutting with ScaI and RsrII. These enzymes cut the genomic RRV clone at nucleotides 9201 and 9568 respectively. Each of these fragments was individually ligated with ScaI/BglII fragment spanning nucleotides 12-1766 and the large RsrII/BglII fragment (resulting from a partial RsrII digest) from nucleotide 2133 to nucleotide 12. These plasmids are designated pRRV-E2E1A-T216R, pRRV-E1E2A-T216V, pRRV-E2E1A-N218R and pRRV-E2E1A-N218V, respectively, according to the single amino-acid change in each glycoprotein coding sequence. The integrity of each cloned plasmid was confirmed by sequence analysis.

4.2. Production of virus by transient expression of envelope glycoproteins

4,000,000 producer ØNXnls*lac*Z cells were grown overnight in 10 cm plates. The following day they were washed with phosphate-buffered-saline solution (PBS), and 10 mL of fresh growth medium was added to them. Plasmids expressing RRV envelope glycoproteins: pRRV-E1E2A, pRRV-E1E2A-T216R, pRRV-E1E2A-N218R, pRRV-E1E2A-T216V and pRRV-E1E2A-N218V were incubated with 12 μL PLUS reagent (Life Technologies) in DMEM for 15 min followed by incubation with 18 μL Lipofectamine (Life Technologies) for 15 min in DMEM at room temperature. Each of these plasmids was then added to the respective plates containing the producer cells grown overnight, and the plates were incubated at 37 °C in 5% CO_2. 3 h after incubation the liquid from each plate was replaced with 10 mL of fresh growth medium. This was further incubated for 48 h at 37 °C in 5% CO_2 after which the supernatant medium containing RRV-MoMLV pseudotyped virus was collected from each of these plates.

4.3. Transduction assay

10 mL of supernatant media overlying producer ØNXnls*lac*Z cells were collected 48 h after transfection and passed through a 0.45 μm filter. Hexadimethrine bromide (5 μg/mL) was added to the filtered supernatant media. These media were used to transduce target cell-lines, NIH 3T3, BHKs, CHO22 and CHO18.4, a mutant cell line derived from the parental CHO22 that does not express HS. After 4 h of incubation, the media were replaced with fresh media. 48 h after transduction, cells were stained with X-gal (Taylor and Sanders, 1999). Transduction units were then calculated by the formula: Transduction Units/ml (TU/mL) = (No. of blue cells/Total number of cells)*(No. of cells plated/Volume of media added).

4.4. Immunoblot assay

10 mL of supernatant media from producer cells transfected with different plasmids were passed through a 0.45 μm filter. The virus particles were collected by ultra-centrifugation of the supernatant media through a 30% sucrose cushion in Beckman 50.2 Titanium rotor at 28000 rpm. The pellets of virus particles accumulated at the bottom were suspended in SDS-PAGE buffer containing 2% β-mercaptoethanol. The viral pellets were analyzed on SDS-PAGE gel and then transferred to nitrocellulose paper. Presence of the envelope glycoproteins RRV-E2 and RRV-E1 along with MoMLV-Capsid (p30) proteins was detected by incubating with rabbit anti-E2, rabbit anti-E1 and goat anti-Raucher Leukemia virus-Capsid (p30) polyclonal primary antibodies (1:5000) respectively. Anti-rabbit antibodies (Chemicon) and anti-goat antibodies coupled with horseradish peroxidase were used as secondary antibodies (1:5000) to detect RRV envelope glycoproteins and MoMLV capsid proteins respectively. The amount of protein present was measured based on chemi-luminescence using FluorChem E. The lysate and cell debris were obtained by lysing the producer ØNXnls*lac*Z cells transfected with different RRV envelope plasmids, using buffer containing 1% Triton-X 100. Immunoblot analysis of these preparations was also done as above.

4.5. Immunofluorescence assay

72 h after transfection of CHO22 and CHO18.4 cell lines with RRV-envelope encoding plasmids, cells were washed with PBS, fixed with 2% formaldehyde and permeabilized with 0.1% Triton-X 100. This was followed by blocking them with 1% BSA solution after which they were incubated overnight at 4 °C with primary Anti-E2 (rabbit) antibodies. After washing off the excess primary antibodies, cells were incubated with Alexa Fluor 488-coupled anti-rabbit antibodies for 1–2 h at room temperature followed by removal of excess antibodies by washing. The cells were visualized by fluorescence microscopy (Olympus IX) using MetaMorph software.

4.6. Addition of heparinase I to producer cells

44 h after transfecting producer ØNXnls*lac*Z cells with RRV envelope plasmids, the supernatant media overlying the cells were replaced with fresh media, and the cells were incubated with 6 μg/mL Heparinase I at 37 °C. After 4 h the supernatant media were collected and passed through a 0.45 μm filter. Hexadimethrine bromide (5 μg/mL) was added to the filtered supernatant media. This media was used to transduce target cell lines (NIH 3T3, BHK, CHO22 and CHO18.4)

4.7. Addition of heparinase I to target cells

Target cells (BHK, CHO22 and CHO18.4) grown overnight were incubated with increasing concentrations (0, 2, 4, 6 μg/mL) of Heparinase I, an enzyme that cleaves HS from the cell surface, for one hour at 37 °C. After washing with PBS, these cells were transduced using supernatant media from producer ØNXnls*lac*Z cells as explained earlier.

4.8. Normalization following quantitative analysis of the immunoblot assay

10 mL supernatant media from 4,000,000 producer cells transfected with different plasmids were collected and replaced with 10 mL fresh media. Collected media were passed through a 0.45 μm filter and subjected to ultracentrifugation in Beckman 50.2 Titanium rotor at 28000 rpm with 30% sucrose cushion to obtain pellets of viral particles. An immunoblot assay of virus pellets using anti-E2 antibodies was performed. Using Image J software, the amount of RRV-E2 glycoprotein in each virus pellet was quantified. Based on these values, the amount of overlying media collected from the producer cells was normalized

across all the samples and used for transduction of target cells (NIH 3T3, BHK, CHO22 and CHO18.4). Normalization was performed by appropriate dilutions of the media containing WT-RRV, T216R-RRV, T216V-RRV, N218R-RRV and N218V-RRV pseudotyped viruses.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.01.022.

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