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Altering the Tropism of Lentiviral Vectors through Pseudotyping

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Abstract

The host range of retroviral vectors including lentiviral vectors can be expanded or altered by a process known as pseudotyping. Pseudotyped lentiviral vectors consist of vector particles bearing glycoproteins (GPs) derived from other enveloped viruses. Such particles possess the tropism of the virus from which the GP was derived. For example, to exploit the natural neural tropism of rabies virus, vectors designed to target the central nervous system have been pseudotyped using rabies virus-derived GPs. Among the first and still most widely used GPs for pseudotyping lentiviral vectors is the vesicular stomatitis virus GP (VSV-G), due to the very broad tropism and stability of the resulting pseudotypes. Pseudotypes involving VSV-G have become effectively the standard for evaluating the efficiency of other pseudotypes. This review samples a few of the more prominent examples from the ever-expanding list of published lentiviral pseudotypes, noting comparisons made with pseudotypes involving VSV-G in terms of titer, viral particle stability, toxicity, and host-cell specificity. Particular attention is paid to publications of successfully targeting a specific organ or cell types.

Keywords

Lentiviral vector; Gene therapy; Glycoproteins; Vector tropism

1. EXPANDING THE TROPISM OF LENTIVIRUSES THROUGH PSEUDOTYPING

One mechanism for expanding the cellular tropism of enveloped viruses is through the formation of phenotypically mixed particles or pseudotypes, a process that commonly occurs during viral assembly in cells infected with two or more viruses [Zavada, 1982]. Human immunodeficiency virus type 1 (HIV-1) has long been known to form pseudotypes by the incorporation of heterologous GPs through phenotypic mixing, allowing an extension of the host range of such virions beyond cells that express the CD4 receptor and an appropriate coreceptor. Several studies have demonstrated that wild type HIV-1 produced in cells infected with xenotropic murine leukemia virus (MLV) [Canivet, 1990; Lusso, 1990], amphotropic MLV [Chesebro, 1990; Spector, 1990], or herpes simplex virus [Zhu, 1990] gave rise to phenotypically mixed virions with an expanded host range, suggesting that pseudotyped virions had been produced. Phenotypic mixing of viral GPs has also been shown to occur between HIV-1 and vesicular stomatitis virus in coinfecting cell cultures [Zhu, 1990]. These early observations were key to the subsequent design of HIV-1-based lentiviral vectors bearing heterologous GPs.
2. PSEUDOTYPED HIV-1 VECTOR PARTICLES

Page et al. [Page, 1990] were the first to design and test HIV-1-based vector particles harboring a heterologous GP. In this study, the HIV-1 genome was rendered replication defective by replacing the GP-encoding gp160 sequence with a guanine-phosphoribosyl transferase (gpt) gene driven by the Simian virus 40 (SV40) early promoter. Transient cotransfection of monkey COS-7 cells with this GP-deleted vector and an expression plasmid encoding an amphotropic MLV GP resulted in packaging of the HIV-gpt vector genome into infectious virions. Upon infection of susceptible cells, the gpt gene was transmitted and expressed, allowing transduced cells to be selected in media containing mycophenolic acid [Page, 1990]. Landau et al. [Landau, 1991] subsequently demonstrated that expression of other GPs including those of the ecotropic MLV and human T-cell leukemia virus type I (HTLV-I) in cells transfected with the HIV-gpt vector resulted in the production of vector pseudotypes capable of infecting murine and human cells with titers reaching $10^5$ colony forming units per ml following mycophenolic acid selection.

3. EARLY LENTIVIRAL VECTORS AND THE RISE OF VSV-G PSEUDOTYPES

These early observations were confirmed and extended in 1996 by results reported independently by three groups showing that VSV-G was also efficiently incorporated into HIV-1 virions [Akkina, 1996; Naldini, 1996; Reiser, 1996]. Lentiviral vectors pseudotyped with VSV-G presented significant advantages in that VSV-G appears to interact with a ubiquitous cellular “receptor” on cells, giving the vector a broad host-cell range. The receptor for VSV has long been thought to be a ubiquitous membrane lipid, phosphatidylserine [Schlegel, 1983]. However, recent experimental evidence has indicated that phosphatidylserine is not the cell surface receptor for VSV, although it may be involved in a postbinding step of virus entry [Coil, 2004]. VSV-G pseudotyping also confers high vector particle stability, allowing for concentration by ultracentrifugation [Burns, 1993; Bartz, 1996]. Perhaps the most significant shortcoming for VSV-G is that it is toxic to cells if expressed constitutively. The use of tetracycline-regulatable promoters has alleviated this problem and facilitated the generation of stable cell lines expressing VSV-G [Ory, 1996]. Another potential drawback for in vivo applications is that VSV-G pseudotypes are inactivated by human serum complement [DePolo, 2000]. This problem can be bypassed by using polyethylene glycol-modified (PEGylated) VSV-G pseudotypes. Covalent modification of VSV-G pseudotyped HIV-1 vector particles using monomethoxypoly(ethylene) reduced the rate of vector inactivation by a factor of 1,000 and significantly enhanced transduction efficiency in the bone marrow and spleen after systemic administration of the virus [Croyle, 2004].

To date, VSV-G remains the most popular GP for pseudotyping HIV-1-based vectors as well as other lentiviral vectors including vectors based on HIV-2 [Poeschla, 1998a] and on simian immunodeficiency virus (SIV) [Nakajima, 2000; Negre, 2000; Schnell, 2000]. Vectors based on nonprimate lentiviruses include feline immunodeficiency virus (FIV) [Poeschla, 1998b], equine infectious anemia virus (EIAV) [Mitrophanous, 1999], bovine immunodeficiency virus (BIV) [Berkowitz, 2001a], Jembrana disease virus (JDV) [Metharom, 2000], visna virus (VV) [Berkowitz, 2001b], and caprine arthritis encephalitis virus (CAEV) [Mselli-Lakhal, 2000] (Table 1).

4. LENTIVIRAL PSEUDOTYPES INVOLVING ALTERNATIVE GLYCOPROTEINS

There is an ever-growing list of alternative GPs for pseudotyping lentiviral vectors, each with specific advantages and disadvantages. While space limitations preclude an exhaustive description of all pseudotypes, this review will survey some of the more prominent examples.
in the recent literature. Other emerging pseudotypes are referred to in Table 1. The widespread use of VSV-G to pseudotype lentiviral vectors has made this GP in effect the standard against which the usefulness of other viral GPs to form pseudotypes are compared. Comparison of pseudotype efficiency inevitably means comparing vector titers on cell lines in vitro. This brings up two important issues: (i) Techniques to determine lentiviral vector titers, and (ii) Cell lines chosen for titration of such vectors. There are different measures used to determine lentiviral vector titers. Some of them are based on the number of vector particles present in a virus stock while others are derived from the number of proviral copies in transduced target cells. Virus particle numbers can be determined using real-time PCR based on strong-stop cDNA present in virions [Scherr, 2001]. Alternatively, the amount of virus proteins present in virus cores such as p24 Gag are determined by ELISA to arrive at relative particle titers [Logan, 2004]. Functional titration assays are based on vector-encoded reporter gene expression. For example, vectors encoding green fluorescent protein (GFP) have been titrated using FACS analysis [Zhang, 2002]. For vectors that do not contain a reporter gene, proviral DNA copy numbers determined by real-time PCR using DNA extracted from transduced cells have been used to determine titers [Sastry, 2002]. As each of these methods produces different results, a reader of the literature must pay close attention to which titration method was used. Also significant for titration is the cell line used as receptors for a given GP may vary from cell line to cell line, possibly producing a falsely depressed titer. Frequently, a given cell line (such as HeLa or 293 cells) is chosen because it is considered generally permissive to infection rather than representing the natural tropism of the virus from which the GP was derived. Additionally, results produced with a given cell line or type of cell in vitro do not always translate into comparable results in vivo due to factors such as complement-mediated inactivation of vectors. Careful attention must also be paid to the given virus strain from which the GP was derived as this may also be a source of confusion and conflicting results. For example, considerable differences in the brain transduction patterns were demonstrated between two related lyssavirus-derived GPs. Transduction of the mouse striatum by HIV-1 vectors pseudotyped with the GP derived from a Mokola virus originally isolated from a cat in Zimbabwe (MokZIM) [Bourhy, 1989] was inefficient [Desmaris, 2001], while HIV-1 vectors bearing a Mokola virus GP obtained from an Ethiopian Mokola virus isolate (MokETH) [Mebatsion, 1995] revealed robust transduction [Watson, 2002].

The following is an overview of prominent examples of lentivirus pseudotypes that have appeared in the literature. Additional examples that are not discussed in the text are referred to in Tables 1 and 2.

4.1. Pseudotypes Bearing Lyssavirus-derived GPs

The capacity of lentiviral vectors to form pseudotypes with lyssavirus GPs was investigated early on with a view toward designing neurotropic lentiviral vectors. The genus Lyssavirus is part of the Rhabdoviridae family and includes the classical rabies viruses and rabies-related viruses including Mokola virus [Badrane, 2001]. Rabies virus (RV), the prototype of the genus Lyssavirus, is an enveloped negative-strand RNA virus encoding five structural proteins including the transmembrane GP. The transmembrane GPs of lyssaviruses appear to exhibit a similar overall structure; however, considerable antigenic differences were noted between RV and rabies-related viruses by using anti-GP monoclonal antibodies. Rabies is a disease of the CNS and neuroinvasiveness is the major defining characteristic of a classical RV infection.

Mochizuki et al. [Mochizuki, 1998] were the first to describe robust pseudotyping of HIV-1 lentiviral vectors using lyssavirus-derived GPs including those of the rabies virus strain RabSADB19 [Conzelmann, 1990] and the Mokola virus MokETH strain [Mebatsion, 1995]. The Lyssavirus pseudotypes were compared to VSV-G pseudotypes by analyzing unconcentrated virus stocks on human osteosarcoma (HOS) cells and on Rat-2 fibroblasts. The
lyssavirus GPs produced identical titers, which were 5 times lower than those of VSV-G pseudotypes on HOS cells and 4 times lower on Rat-2 cells. Shortly thereafter, EIAV vectors were successfully pseudotyped with a RV GP [Mitrophanous, 1999] (the source of the rabies GP used for pseudotyping was not revealed).

4.1.1. Neurotropism of Lentiviral Vectors Pseudotyped with Lyssavirus GPs—

Once successful pseudotyping of lentiviral vectors with lyssavirus-derived GPs had been demonstrated, interest was focused on assessing the neurotropism of such pseudotypes. Desmaris et al. [Desmaris, 2001] initially tested the infectivity of HIV-1 vector pseudotypes bearing a Mokola virus GP (MokZIM strain) or VSV-G. The in vivo tropism of the two pseudotypes was tested by injecting vector particles encoding β-galactosidase (β-gal) into the striatum. The two pseudotypes produced comparable numbers of β-gal expressing cells, and co-labeling for cell-type markers indicated that both vectors had primarily transduced neurons. Watson et al. [Watson, 2002] described a similar analysis involving HIV-1 vectors pseudotyped with the MokETH GP. Intrastriatal injection of such pseudotypes in the mouse also resulted in extensive transgene expression along the corpus callosum and external capsule. Transduction by pseudotypes bearing the MokETH GP in both the striatum and the white matter was considerably more extensive than that observed with pseudotypes involving VSV-G. Mokola pseudotypes however were not transported along projections from the striatum to the substantia nigra [Watson, 2002]. To assess whether lentiviral vector particles pseudotyped with lyssavirus GPs gain access to the CNS by retrograde transport, pseudotyped HIV-1 virions were either injected into the nasal cavity or injected into limb muscles of rats. Both VSV-G and MokZIM pseudotypes produced β-gal-expressing neurons in the olfactory bulb after nasal injection, but neither produced β-gal-positive motor neurons in the spine [Desmaris, 2001]. The investigators concluded that VSV-G and MokZIM pseudotypes were of comparable efficiency in retrograde axonal transport, but that neither was taken up at the neuromuscular junction. In a related study, EIAV-based vectors pseudotyped with the GPs derived from the RV RabERA strain [Sacramento, 1992] or from VSV-G were injected into the striatum to compare the spread of the virus as judged from the number and distribution of transgene-expressing cells [Mazarakis, 2001]. After injection of VSV-G pseudotypes, some 30,000 β-gal-positive cells were found in the striatum. At a comparable titer, RabERA pseudotypes produced 32,000 β-gal-positive cells in the striatum plus 14,800 cells in the thalamus along with 3000 cells in the substantia nigra, indicating both retrograde and anterograde transport. The GPs of the rabies RabCVS [Sacramento, 1992] and RabERA strains produced similar transgene expression patterns in distal brain areas after striatal injection of EIAV vectors [Wong, 2004]. However, a change from arginine to glutamine at amino acid 333 of the RabERA GP resulted in weaker transduction of the caudate putamen. Interestingly, there was no retrograde transport of vectors bearing the mutated RabERA GP to areas such as the thalamus. This suggests that this single mutation in the viral GP was sufficient to abolish the virus’ retrograde transport characteristics [Wong, 2004]. Intraspinal injections of EIAV vectors pseudotyped with the RabERA GP have likewise demonstrated spread of transgene expression along nerve tracts. This was not seen after injection of VSV-G pseudotypes [Mazarakis, 2001].

4.1.2. Lentiviral Vectors Pseudotyped with Lyssavirus GPs to Treat Motor Neuron Disease—

Of potential clinical importance, intramuscular injection of EIAV vectors pseudotyped with RV GPs resulted in transgene expression in spinal motor neurons. Retrograde transport of lentiviral vectors from peripheral muscle to spinal motor neurons has been used to demonstrate a therapeutic effect in an animal model of familial amyotrophic lateral sclerosis (ALS) [Azzouz, 2004b]. This model is based on SOD1<sup>G93A</sup> mice which have a mutation in the SOD1 gene and experience severe motor neuron degeneration comparable to that seen in human ALS. EIAV-based vectors encoding vascular endothelial growth factor (VEGF) were
pseudotyped with the RabERA GP and injected into various muscle groups of 3 week-old SOD1\(^{G93A}\) mice. Animals that were injected with a control vector were severely impaired by 100 days of age, while comparable impairment in animals that received the VEGF vector was delayed until a month later. EIAV/VEGF vector treatment also extended lifespan of these animals by 30%. Clinical markers such as rotarod performance were also improved with EIAV/VEGF vector treatment. In a related study, EAIV vectors expressing either a marker gene or the human survival motor neuron (SMN) cDNA and pseudotyped with the RabERA GP were injected into various muscles of spinal muscle atrophy (SMA) mice. Retrograde transport of the virus to spinal motor neurons was confirmed by marker gene expression. Compared to untreated controls, SMN-vector treatment restored SMN protein levels in motor neurons, reduced motor neuron death, and increased the life expectancy by an average of 3 and 5 days (20% and 38%) [Azzouz, 2004a]. These examples demonstrate the capacity of lentivirus vectors pseudotyped with RV GPs to deliver therapeutic genes to otherwise inaccessible neurons by exploiting the lyssavirus’ ability to induce retrograde transport along axons.

4.2. Pseudotyped Lentiviral Vectors Bearing Lymphocytic Choriomeningitis Virus GPs

4.2.1. Pseudotype Formation Using Lymphocytic Choriomeningitis Virus GPs—MLV-based retroviral vectors pseudotyped with the lymphocytic choriomeningitis virus (LCMV) GP have been shown to efficiently transduce cells from different tissues relevant to gene therapy including fibroblasts, epithelial cells, hematopoietic cells, as well as hepatoma, neuroblastoma and glioma cell lines [Miletic, 1999]. Successful transduction involving pseudotypes containing the LCMV-WE GP [Romanowski, 1985] was observed in cell lines derived from various species of human, mouse, hamster, or dog origin. LCMV is a noncytopathic arenavirus, which infects a broad range of different cell types. The LCMV GPs are synthesized as a 74-kDa precursor protein, GP-C, and then cleaved into a 35-kDa transmembrane protein, GP-2, and an external 44-kDa protein, GP-1. Alpha-dystroglycan, a cellular receptor for LCMV [Cao, 1998; Smelt, 2001], is a dystrophin-associated GP that connects the cytoskeleton with the extracellular matrix, and is widely expressed in most tissues.

Pseudotyping of HIV-1-based lentiviral vectors with the GP derived from the LCMV Armstrong 53b strain (LCMV-Arm53b, [Salvato, 1988]) was first reported in 2001 [Christodoulopoulos, 2001]. The titers obtained with such pseudotypes on 293T cells were at least two logs below those observed with vectors pseudotyped with the amphotropic MLV GP or VSV-G. Interestingly, LCMV-pseudotyped lentiviral vectors containing the GP of the LCMV-WE strain revealed vector titers similar to those obtained with pseudotypes containing the amphotropic MLV GP or VSV-G [Beyer, 2002].

4.2.2. Applications of LCMV Pseudotypes—A number of applications involving lentiviral vectors pseudotyped with LCMV GPs have been described. The following is an overview of these applications. Loss of insulin-secreting pancreatic islet cells leads to insulin-dependent diabetes. As diabetes is a significant public health problem, genetic manipulation of islet cells aimed at preventing their destruction is important for both clinical applications and understanding islet cell biology. Kobinger \textit{et al.} [Kobinger, 2004] evaluated the ability of a HIV-1-based lentiviral vector pseudotyped with various viral GPs to target human islets ex vivo, with the goal of improving transduction efficiency while minimizing toxicity. LCMV-pseudotyped vectors transduced insulin-secreting beta cells with the highest efficiency. Moreover, toxicity associated with transduction of islets was found to be lower with LCMV-WE-pseudotyped vectors than with VSV-G-pseudotyped vectors, the second most efficient pseudotype for islet transduction.

A reduced toxic response to LCMV pseudotypes compared to VSV-G pseudotypes was also found in the liver following portal vein injection of HIV-1-based vectors encoding human
blood-clotting factor VIII (hFVIII) [Park, 2003]. Transduction of hemophilia A mice with hFVIII expressing lentiviral vectors resulted in transient correction of the bleeding diathesis phenotype. Moreover, the use of vectors pseudotyped with the LCMV-WE GP resulted in similar circulating levels of hFVIII. Similar doses of LCMV-pseudotyped lentiviral vectors resulted in minimal systemic or hepatic injury compared to VSV-G pseudotypes. Taken together, these studies demonstrate the importance of using alternative pseudotypes, such as LCMV, to minimize adverse vector effects in vitro and in vivo.

There is increasing interest in developing gene therapy strategies for malignant gliomas, which are the most frequent primary brain tumors, but have a poor prognosis with current treatments. Work reported by Beyer et al. [Beyer, 2002] showed that LCMV-WE pseudotypes were able to transduce human glioma and neuroblastoma cell lines, as well human primary glioma cells derived from resected human gliomas. In a related study, Miletic et al. [Miletic, 2004] assessed the transduction efficiencies of HIV-1 vectors pseudotyped with the LCMV-WE GP or VSV-G in malignant glioma cells and normal brain cells both in vitro and in vivo. In vitro, LCMV-WE pseudotypes transduced almost exclusively astrocytes, whereas VSV-G pseudotypes transduced neurons as well as astrocytes. In an in vivo glioblastoma model based on rats carrying intracerebral grafts of rat 9L gliosarcoma cells, LCMV-WE pseudotypes revealed efficient transduction of solid glioma parts and specific transduction of infiltrating tumor cells. In contrast, VSV-G-pseudotyped lentiviral vectors transduced only a few tumor cells in solid tumor parts and transduced mostly neurons surrounding the tumors. Similar results were reported by Steffens et al. [Steffens, 2004]. These authors compared eight different lentivirus pseudotypes regarding their efficiencies to transduce neuronal and glial tumor cell lines. Pseudotypes involving the GPs of human foamy virus, RV (RabSADB19 strain), Mokola (MokETH strain) or amphotropic MLV displayed the highest transduction efficiency in neuroblastoma cells, whereas pseudotypes involving the LCMV-Arm53b GP resulted in the highest transduction efficiency in gliomas.

In a recent report, an FIV vector expressing β-gal and pseudotyped with the LCMV-WE GP was injected into postnatal mouse brain striatum to determine neural cell-type transduction [Stein, 2005]. β-gal-expressing cells included astrocytes in the striatum and in the subventricular zone (SVZ), neuroblasts along the rostral migratory stream, and neurons in the olfactory bulb. This pattern was suggestive of transduction of neural stem/progenitor cells that reside in the SVZ and continually generate olfactory bulb neurons. Thus, FIV/LCMV-WE pseudotypes are potentially useful for targeting neural stem/progenitor cells in vivo. Taken together, these results demonstrate that targeted transduction in the CNS can be achieved using specific pseudotypes.

4.3. Lentivirus Pseudotypes Bearing Alphavirus GPs

Alphaviruses exhibit a wide cellular tropism that includes important gene therapy targets such as antigen-presenting cells, neurons, and muscle cells. Alphaviruses belong to the family Togaviridae and are found in insects, reptiles, birds, and mammals. Although it remains to be determined as to what molecules act as cellular receptors for these viruses, it has been suggested that several receptors or several receptor-coreceptor combinations are involved in virus entry. The main part of the viral GP complex consists of heterodimers of the E1 and E2 GPs that are further organized into trimers. A total of 80 of these trimeric spikes are distributed on the outer surface of the virion. The E2 GP most likely mediates interactions with target cell receptors, whereas E1 is thought to mediate fusion. Several reports describing successful pseudotyping of lentiviral vectors involving the Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus GPs have appeared in the literature.
4.3.1. Lentiviral Vectors Pseudotyped with the RRV and SFV GPs—Kang et al. [Kang, 2002] were the first to describe lentivirus pseudotypes involving alphavirus GPs. These authors examined the in vivo gene transfer efficiency and tissue or cell tropism of a FIV-based lentiviral vector pseudotyped with the GPs derived from RRV. RRV GPs were efficiently incorporated into FIV virions, generating preparations of FIV vectors, which after concentration attained high titers. After systemic administration, RRV-pseudotyped FIV vectors (RRV/FIV) predominantly transduced the liver of recipient mice. Transduction efficiency in the liver with the RRV/FIV was about 20-fold higher than that achieved with a VSV-G pseudotype. Moreover, in comparison to VSV-G, the RRV GPs caused less cytotoxicity. Although hepatocytes were the main liver cell type transduced, nonhepatocytes (mainly Kupffer cells) were also transduced. After injection into the brain, RRV/FIV preferentially transduced neuroglial cells (astrocytes and oligodendrocytes). In contrast to the VSV-G protein that targets predominantly neurons, <10% of the brain cells transduced with the RRV pseudotyped vector were neurons.

Kahl et al. [Kahl, 2004] subsequently showed that the GPs derived from RRV and SFV can pseudotype HIV-1-derived lentivirus vectors and that both RRV and SFV GPs considerably expanded the host range of these vectors. Also, such pseudotypes could be efficiently concentrated by ultracentrifugation. A systematic analysis comparing the alphaviral GPs to VSV-G revealed that lentivirus vectors incorporated RRV GPs with an efficiency comparable to that of VSV-G. Both pseudotypes had comparable physical titers as judged by quantitative RT-PCR amplification of vector RNA. However, infectious titers with the RRV pseudotype were lower than with VSV-G as determined by FACS analysis of GFP expression in transduced cells. However, incorporation of SFV GPs into lentivirus vector was less efficient, leading to significantly decreased physical and infectious titers.

In a recent report by Kahl et al. [Kahl, 2005] the transduction of hematopoietic cells using RRV-pseudotyped lentiviral was investigated. RRV-mediated transduction of human CD34+ cord blood cells and progenitors was very inefficient, even at multiplicities of infection of 100. Inefficient transduction was also observed in a variety of hematopoietic cell lines. However, exposing the monocytic cell line THP-1 to 12-O-Tetradecanoylphorbol-13-acetate (TPA), a very potent activator of macrophage differentiation, induced a more than 10-fold increase in transduction. The uniquely restricted host range of RRV-pseudotyped vectors in hematopoietic cells may aid in the design of novel cell-selective transduction strategies.

Sustained production of lentiviral vectors pseudotyped with RRV and SFV from stable packaging cell lines was reported by Strang et al. [Strang, 2005]. Cell clones producing high-titer RRV pseudotypes were stable for at least 5 months while clones producing SFV pseudotypes produced relatively low titers. RRV pseudotypes were found to display a number of characteristics that are attractive, including resistance to inactivation by heat-labile components in fresh human sera and thermostability at 37°C. Upon concentration by ultracentrifugation, vector stocks with titers up to $6 \times 10^7$ transducing units (TU)/ml were obtained.

4.3.2. Lentiviral Vectors Pseudotyped with Sindbis Virus GPs—One strategy for cell-specific targeting has been the insertion of cell-specific ligands including single chain antibodies into the viral GP, or to bridge the virus and target cell using a ligand or antibody bridge. Morizono et al. [Morizono, 2001] having shown that the GP of Sindbis virus is able to pseudotype oncoretroviruses and lentiviruses, reported an oncoretroviral and lentiviral gene-targeting system based on antibody-mediated specific binding of a modified chimeric Sindbis virus GP. The Fc-binding domain (ZZ domain) of protein A was inserted into the Sindbis E2 protein, to produce the ZZ.SINDBIS GP. They also showed that monoclonal antibodies directed to cell-surface antigens can be used to redirect the target specificity of these vectors when
pseudotyped with the modified Sindbis virus GP. Of particular note, the vectors maintained high viral titers, which could be further increased by simple ultracentrifugation. Intravenous injection of such pseudotypes into mice resulted in high levels of transduction in liver and spleen cells. An exciting recent report from the same lab [Morizono, 2005] documented successful targeting in a living animal through intravenous injection of a lentiviral vector pseudotyped with a modified chimeric Sindbis virus GP. The modified GP (termed m168) contained a ZZ domain and carried a 4 aa deletion in E3 and two separate point mutations within E2. m168 pseudotypes were found to display a high targeting specificity and, unlike other pseudotypes, had low nonspecific infectivity in liver and spleen. A mouse cancer model of metastatic melanoma was used to test intravenous targeting with m168. Human P-GP was ectopically expressed on the surface of melanoma cells and targeted by the m168 pseudotyped lentiviral vector conjugated with antibody specific for P-glycoprotein. m168 pseudotypes successfully targeted metastatic melanoma cells growing in the lung after systemic administration by tail vein injection. Further development of this targeting technology may result in applications not only for cancers but also for genetic, infectious and immune diseases.

4.4. Pseudotypes Bearing Filovirus GPs

4.4.1. Pseudotype Formation Using Filovirus GPs—In 2000 Chan et al. [Chan, 2000] described the successful pseudotyping of HIV-1 vectors using Marburg virus and Ebola Zaire (EboZ) virus-derived virion GPs. The folate receptor-alpha was soon thereafter identified as a cofactor for cellular entry by Marburg and Ebola viruses [Chan, 2001]. The Ebola and Marburg filoviruses are highly pathogenic viruses that induce hemorrhagic fevers in humans and nonhuman primates. Of the four identified strains of Ebola virus, Zaire, Ivory Coast, Sudan, and Reston, the Zaire strain induces the highest death rates in humans, while the Reston strain has not caused fatal disease in humans. Mononuclear phagocytic cells form the primary targets for filovirus replication, while endothelial cells serve as secondary targets. The GP-encoding region of Ebola virus gives rise to two products, a nonstructural secreted form of the GP, sGP, encoded by the predominant transcript (80%), and the virion GP, the result of RNA editing during formation of the message (20%).

4.4.2. In Vivo Tropism of Lentiviral Vectors Pseudotyped with the EboZ GP—The in vivo tropism of lentiviral vectors pseudotyped with EboZ as compared to VSV-G was tested by in utero intramuscular and intrahepatic injections [MacKenzie, 2002]. Injected animals were harvested at time points between 5 days and 9 months following injection and detailed histologic assessments were performed. The efficiency and distribution of transduction after in utero administration was highly dependent upon the route of administration and the vector pseudotype used. Biodistribution studies showed widespread distribution of vector sequences in multiple tissues, albeit at very low levels, and transduced cells were found in significant numbers only in liver, heart, and muscle. Overall, vectors pseudotyped with VSV-G were the most efficient in transducing hepatocytes, whereas vectors pseudotyped with the MokETH and EboZ GPs were more efficient in transducing myocytes. Transduction of cardiomyocytes was observed after both intramuscular and intrahepatic injection of all three vectors. EboZ-pseudotyped vectors injected into muscle in utero were found to transduce muscle satellite or stem cells. Examination of transgene expression in regenerated muscle after injury showed that the newly generated tissue had originated from transduced satellite cells [MacKenzie, 2005]. Direct brain injection of EboZ/HIV pseudotypes in adult mice failed to transduce cells in the brain [Watson, 2002] and EboZ pseudotypes were also unable to transduce oligodendrocyte cultures. This may be due to the lack of the proper receptor since folate receptor-alpha expression was found to be low in the CNS.

4.4.3. Airway Applications Involving Lentiviral Vectors Pseudotyped with Filovirus GPs—The application of gene delivery strategies as a treatment for cystic fibrosis...
is limited by poor gene transfer efficiency with vectors applied to the apical surface of airway epithelia. To overcome this limitation, Kobinger et al. [Kobinger, 2001] carried out a systematic analysis of lentivirus vector pseudotypes to stably transduce airway epithelia in vivo. To do this, HIV-1-based vectors pseudotyped with the amphotropic MLV, Mokola (MokETH), HA (influenza-hemagglutinin), EboZ and Ebola-Reston (EboR) GPs were applied apically and basolaterally to cultures of airway epithelial cells. EboR and HA pseudotypes were completely ineffective, and only EboZ pseudotypes were able to efficiently transduce after apical application. In the same study, human tracheal explants were incubated with EboZ and VSV-G pseudotypes. VSV-G was largely ineffective while the EboZ pseudotype efficiently transduced airway epithelium. EboZ pseudotypes were similarly more effective than VSV-G pseudotypes in an in vivo test in mice receiving vector pseudotypes intratracheally. Sinn et al. [Sinn, 2003] found that polarized human airway epithelia expressed abundant folate receptor alpha on their apical surface. In an attempt to target these apical receptors, FIV-based vectors were pseudotyped using GPs derived from the Marburg and EboZ viruses. Deletion of a heavily O-glycosylated extracellular domain of the EboZ GP including amino acids 309 to 489 resulted in the EboZΔO GP variant. The titer of concentrated FIV vector stocks pseudotyped with EboZΔO was increased some 74-fold. Both EboZ/FIV and EboZΔO/FIV transduced airway epithelia from the apical surface at greater efficiency than from the basolateral surface. In contrast, VSV-G/FIV transduced the basolateral surface more efficiently than the apical one. In addition, the EboZ GP contains a mucin-rich region that was demonstrated to reduce expression of cellular adhesion molecules and induce cytotoxic effects to endothelial cells in vitro and in vivo. Medina et al. [Medina, 2003] developed several mutant EboZ GPs that contained deletions corresponding to the mucin-rich domain. Lentiviral vectors pseudotyped with these mutant GPs possessed higher titers compared to vectors pseudotyped with the unmodified EboZ GP. Also, they resulted in more efficient gene transfer in vivo.

4.5. Lentiviral Vectors Bearing Retrovirus GPs

4.5.1. Pseudotype Formation Using Gammaretrovirus GPs—A variety of different envelope GPs derived from gammaretroviruses were shown to pseudotype lentiviruses vectors. The GPs included those derived from the ecotropic MLV [Landau, 1991; Reiser, 1996], amphotropic 4070A MLV [Reiser, 1996], 10A1 MLV [Stitz, 2000; Christodoulopoulos, 2001], xenotropic NZB MLV and that of the polytropic mink cell focus-forming virus [Christodoulopoulos, 2001]. Other GPs included that of the gibbon ape leukemia virus (GALV) [Stitz, 2000; Christodoulopoulos, 2001] and the RD114 GP [Hanawa, 2002; Sandrin, 2002; Zhang, 2004]. The titers of lentiviral vectors pseudotyped with these GPs were usually lower than those observed with VSV-G. However these pseudotypes were stable and retained full infectivity during concentration by ultrafiltration or ultracentrifugation [Reiser, 1996; Reiser, 2000; Hanawa, 2002; Sena-Esteves, 2004].

4.5.2. Lentiviral Vectors Pseudotyped with Gammaretrovirus GPs in Applications Involving CD34+ Hematopoietic Progenitor Cells, T Lymphocytes, and Mesenchymal Stem Cells—Hanawa et al. [Hanawa, 2002] showed that HIV-1 vectors pseudotyped with the RD114 and amphotropic MLV GPs were more efficient than VSV-G pseudotypes at transducing human cord blood-derived CD34+ cells and clonogenic progenitors. In addition, amphotropic MLV pseudotypes transduced cytokine-mobilized, human peripheral blood CD34+ cells capable of establishing hematopoiesis in immunodeficient mice more efficiently than VSV-G pseudotypes. Work reported by Muhlebach et al. [Muhlebach, 2003] revealed that MLV vectors pseudotyped with the MLV 10A1 and the GALV GPs resulted in efficient transduction of preactivated human primary T lymphocytes while pseudotypes bearing the amphotropic MLV, RD114 and VSV-G GPs were less efficient. In contrast, HIV-1 vectors pseudotyped with the same GPs transduced
preactivated T lymphocytes with similar efficiencies. This may reflect differences in the half-lives of lentiviral vector pseudotypes compared to MLV pseudotypes containing the same GPs.

In a study involving pseudotyped SIVmac251-based vectors, Sandrin et al. [Sandrin, 2002] showed that GALV and RD114 pseudotypes displayed a much reduced infectivity relative to vectors pseudotyped with the VSV-G, LCMV and amphotropic 4070A MLV GPs. Interestingly, SIV vectors pseudotypes bearing chimeric GPs with the extracellular and transmembrane domains of GALV or RD114 fused to the cytoplasmic tail of the amphotropic 4070A MLV GP, augmented the transduction efficiency of such pseudotypes in human and macaque primary blood lymphocytes and CD34+ cells. This may have been caused by changes in the intracellular trafficking and assembly of the GPs on viral cores due to changes of the cytoplasmic tails [Sandrin, 2004]. Similar results were reported by Zhang et al. [Zhang, 2004] who showed that transduction efficiencies in human mesenchymal stem cells (MSCs) with HIV-1 vectors pseudotyped with the unmodified RD114 GP were 1 to 2 orders of magnitude below those observed with VSV-G pseudotypes. However, HIV-1 vectors pseudotyped with a chimeric RD114 GP harboring an amphotropic 4070A MLV cytoplasmic tail revealed about 15-fold higher titers relative to the unmodified RD114 GP.

4.6. Lentiviral Vector Pseudotypes Containing the Baculovirus GP64 GP

Baculoviruses, which are used as biopesticides, primarily infect members of the Lepidopteran family. It was found that recombinant baculoviruses are able to deliver transgenes into mammalian cells provided that they are controlled by a promoter that is active in mammalian cells [Ghosh, 2002]. GP64 is the major baculovirus GP and interest has recently been directed towards using the GP64 GP to pseudotype lentiviral vectors [Kumar, 2003]. GP64-pseudotyped HIV-1 vectors tolerated ultracentrifugation well and efficiently transduced various cell types although GP64 pseudotypes showed a more restricted tropism than VSV-G pseudotypes, with an especially poor ability to transduce hematopoietic cell types including dendritic cells [Schauber, 2004]. More notably, GP64 expression was not cytotoxic to host cells. Thus, derivation of a permanent cell line constitutively expressing GP64 at high levels over many passages was successful [Kumar, 2003]. Lentiviral vectors pseudotyped with the baculovirus GP64 were found to be inactivated by human complement [Schauber, 2004]. In animal sera, the vectors were mostly resistant to inactivation by rodent complement, whereas canine complement caused a moderate reduction in titer [Schauber-Plewa, 2005]. Human complement-resistant pseudotyped HIV-1 vector particles were subsequently produced through incorporation of complement regulatory proteins. Co-display of decay accelerating factor (DAF)/CD55 or of a GP64-DAF fusion protein stabilized GP64-bearing vector particles against inactivation by human and nonhuman primate serum [Guibinga, 2005; Schauber-Plewa, 2005].

5. METHODS TO PRODUCE, CONCENTRATE AND PURIFY LENTIVIRAL VECTOR PSEUDOTYPES

5.1. Production of Lentiviral Vectors Using Packaging Cell Lines

To facilitate large-scale reproducible lentiviral vector production, a number of groups have established lentivirus packaging cell lines. Several of the proteins required to assemble lentivector particles including VSV-G [Burns, 1993], HIV-1 Vpr [Bartz, 1996], Tat [Li, 1995], Rev [Miyazaki, 1995], and protease [Konvalinka, 1995] are detrimental to mammalian cells when overexpressed. Thus, for stable production of HIV-1 vectors pseudotyped with VSV-G, 293-based cell lines allowing conditional production of virus-encoded proteins and VSV-G using tetracycline regulated promoters were generated that yielded titers up to $10^6$ TU/ml [Kafri, 1999; Klages, 2000; Farson, 2001; Xu, 2001]. Inducible cell lines for the production of SIV vectors pseudotyped with VSV-G were also established [Kuate, 2002]. This was
achieved with expression plasmids containing a codon-optimized gag-pol coding region of SIV and the VSV-G coding region under the control of a ponasterone-inducible promoter. Vector titers at around $10^5$ TU/ml were obtained.

To rapidly and reproducibly generate high-titer HIV-1 vector pseudotypes, Ikeda et al. [Ikeda, 2003] generated stable cell lines expressing GPs of gammaretroviruses including the amphotropic 4070A MLV GP, the RD114 GP with an HIV protease site introduced at the R-peptide cleavage site (RDpro), or the GALV glycoprotein with an MLV cytoplasmic tail. In concert with a codon-optimized HIV-1 gag-pol coding region, these producer cells could make lentiviral pseudotyped particles with titers up to $10^7$ TU/ml (20 TU/cell/day) for at least three months in culture. Compared to vectors pseudotyped with VSV-G, the gammaretrovirus pseudotypes were relatively stable at 37°C and were resistant to inactivation by freeze/thaw cycling or incubation with human sera [Strang, 2004]. More recently, Strang et al. [Strang, 2005] established stable producer cell lines for HIV-1 vectors pseudotyped with the alphavirus RRV and SFV E2E1 GPs. Stable clones produced high-titer RRV-pseudotyped HIV-1 vectors for at least 5 months. Clones producing SFV pseudotypes, however, revealed relatively low titers.

5.2. Concentration and Purification of Lentiviral Vector Pseudotypes

Typical titers of lentiviral vectors pseudotyped with VSV-G obtained by transient transfection range from $10^6$ to $10^7$ TU/ml. Increased titers can be achieved by physical concentration including ultracentrifugation and ultrafiltration of virus-containing cell culture supernatants. Using a protocol initially designed for the generation of high-titer MLV vector stocks [Burns, 1993], pseudotyped lentiviral particles containing VSV-G have proven to be stable and able to withstand concentration by ultracentrifugation without significant loss in titer [Bartz, 1996]. This greatly facilitated the generation of highly concentrated vector stocks for in vivo applications. Lentiviral vectors harboring a wide variety of different GPs have since been shown to lend themselves to concentration by ultracentrifugation [Reiser, 2000; Kobinger, 2001; Sena-Esteves, 2004].

Concentration protocols based on ultrafiltration were also established for HIV-1-based vectors. Facile ultrafiltration procedures were developed early on [Reiser, 1996] to concentrate HIV-1 pseudotypes. Good recoveries were observed with HIV-1 particles containing VSV-G or the amphotropic MLV GP. Improved procedures for concentration by ultrafiltration of other pseudotypes were subsequently reported [Reiser, 2000; Cui, 2002; Hanawa, 2002; Coleman, 2003; Sena-Esteves, 2004].

Vector production for large-scale in vivo applications that require high-titer stocks is challenging due to the lack of simple procedures capable of rapidly processing large volumes of cell culture supernatant. The traditional ultracentrifugation-based approaches are limited in terms of their capacity to handle large volumes, thus making this procedure extremely tedious. Centrifugation at low speed overnight has been used to process volumes in excess of one liter [VandenDriessche, 2002]. However, this low speed approach is time consuming. Thus, there is an emerging need for quick, reproducible and less laborious procedures that rapidly reduce the volume of the cell culture supernatant to be processed. Pham et al. [Pham, 2001] have described a gentle and simple precipitation method to rapidly concentrate pseudotyped lentiviral vectors involving the co-precipitation of pH-adjusted viral supernatants with calcium phosphate, low speed centrifugation, and dialysis. Volumes were decreased from 300 ml to 10 ml, with 50-100% recovery of infectious virus. Zhang et al. [Zhang, 2001] have presented an alternative precipitation method based on poly-L-lysine. Viral supernatants were mixed with poly-L-lysine, incubated for 30 min at 4°C, and then centrifuged at 10,000 g for 2 h. Recovery of infectious virus ranged from 26-32% after processing up to 3 liters of cell culture supernatant.
One problem with the methods outlined above is that cell-derived components are concentrated along with the vector particles leading to potential immune and inflammation responses [Baekelandt, 2003]. Thus, ultracentrifugation/ultrafiltration and precipitation approaches generate rather impure virus preparations and additional steps including chromatography-based approaches are needed in order to purify the virus of contaminating host cell components. Methods based on anion exchange chromatography of HIV-1 vectors pseudotyped with VSV-G have been established [Scherr, 2002; Yamada, 2003]. Schauber et al. [Schauber, 2004] described a similar procedure for HIV-1 vectors pseudotyped with the baculovirus GP64 GP. Yields and purity of the virus stocks resulting from these procedure were not reported, but these approaches may lead to vector stocks of improved purity, increased infectivity and reduced toxicity.

VSV-G-pseudotyped lentiviral vectors recently entered clinical application. The production, purification and testing protocols were adapted to GMP standards, and eventually gained FDA clearance [Lu, 2004; Manilla, 2005].

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**ACKNOWLEDGMENTS**

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### Table 1. Overview of Lentivirus Pseudotypes

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Vector</th>
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<td>CAEV</td>
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### Table 2.

Cell and Organ Preferences of Lentivirus Pseudotypes

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<td>[Sinn, 2003]</td>
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<td>SeV F and HN</td>
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<td>Targets astrocytes</td>
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<td>Targets neural progenitor/stem cells</td>
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</tr>
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