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Oncoretrovirus and Lentivirus Vectors Pseudotyped with Lymphocytic Choriomeningitis Virus Glycoprotein: Generation, Concentration, and Broad Host Range

Winfried R. Beyer,[1]* Manfred Westphal,[2] Wolfram Ostertag,[1] and Dorothee von Laer[3]*

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg[1] and Neurochirurgische Klinik, Universitätskrankenhaus Eppendorf,[2] D-20251 Hamburg, and Georg-Speyer-Haus, 60596 Frankfurt,[3] Germany

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Lymphocytic choriomeningitis virus (LCMV) is a noncytopathic arenavirus shown to infect a broad range of different cell types. Here, we combined the beneficial characteristics of the LCMV glycoprotein (LCMV-GP) and those of retroviral vectors to generate a new, safe, and efficient gene transfer system. These LCMV-GP pseudotypes were systematically compared with vectors containing the widely used amphotropic murine leukemia virus envelope (A-MLVenv) or the vesicular stomatitis virus G protein (VSV-G). Production of LCMV-GP-pseudotyped oncoretroviral and lentiviral vectors by transient transfection resulted in vector titers similar to those with A-MLVenv or VSV-G. In contrast to A-MLVenv particles, LCMV-GP pseudotypes could be efficiently concentrated by ultracentrifugation without loss of vector titer. Unlike the cell-toxic VSV-G, a stable retroviral packaging cell line constitutively expressing LCMV-GP could be established. Vectors pseudotyped with LCMV-GP efficiently transduced many cell lines from different species and tissues relevant for gene therapy. Transduction of human glioma cells was studied in detail. These cells are a major target for cancer gene therapy and were transduced more efficiently with LCMV-GP-pseudotyped vectors than with the generally used A-MLVenv particles. The high stability, low toxicity, and broad host range make LCMV-GP-pseudotyped vectors attractive for gene transfer applications. The recombinant LCMV-GP-pseudotyped vectors will also allow functional characterization of naturally occurring and recombinant LCMV-GP variants.

Improved gene transfer techniques have been instrumental for the progress toward human gene therapy. The most commonly used transfer vectors in clinical applications are derived from oncoretroviruses (1). These vectors can provide stable gene transfer and expression in mammalian cells, and packaging cell lines have been developed, which safely produce replication-incompetent vectors. A major factor that determines the host ranges of such vectors is the retroviral glycoprotein in the outer lipid envelope of the vector particle. Exchange of the parental ecotropic glycoprotein to the amphotropic murine leukemia virus envelope protein (A-MLVenv) or envelope proteins from other retroviruses can broaden the host range without loss of the beneficial characteristics of the vector system (8, 19, 23). However, because of the instability of the retroviral envelope, the particles cannot be efficiently concentrated to higher titers by ultracentrifugation, limiting the application of retroviral vectors, especially in vivo. In addition, the host range of retroviral vectors is restricted by expression of the cognate cellular receptor, leading to inefficient transduction in some cell types. Both the stability and host range of retroviral vectors have been improved by replacement of the retroviral envelope protein by the rhabdoviral G protein of the vesicular stomatitis virus (VSV-G) (5, 13, 34).

* Corresponding author. Mailing address for Winfried R. Beyer: Heinrich-Pette-Institut, Martinistr. 52, D-20251 Hamburg, Germany. Phone: 49-40-48051-270. Fax: 49-40-48051-187. E-mail: beyer@hpi.uni-hamburg.de. Mailing address for Dorothee von Laer: Georg-Speyer-Haus, Paul-Ehrlich-Str. 42-44, D-60596 Frankfurt, Germany. Phone: 49-69-63395-232. Fax: 49-69-63395-297. E-mail: laer@em.uni-frankfurt.de.

Efficient gene transfer of oncoretroviral vectors is dependent on cell division (20). In contrast, gene transfer systems derived from the lentiviral group of retroviruses can also transduce nondividing cells and can be pseudotyped by VSV-G (21). However, a major drawback of the VSV-G pseudotyped oncoretroviral and lentiviral packaging systems is the toxicity of VSV-G (5, 7).

We previously showed that oncoretroviral vectors derived from the murine leukemia virus (MLV) can be packaged by replication-competent lymphocytic choriomeningitis virus (LCMV). LCMV is a noncytopathic arenavirus, which infects various cell types (14, 31). When aiming to establish recombinant packaging systems for MLV(LCMV) pseudotypes, we found that no cell surface expression of recombinant LCMV glycoproteins (LCMV-GP) was detectable when using the originally published cDNA clone. However, cell surface expression was possible after introduction of a single mutation into the original LCMV-GP cDNA clone (2, 18). LCMV-GP is initially expressed as a precursor polypeptide, GP-C, which is posttranslationally processed by a cellular protease into GP-1 and GP-2. GP-1 meditates binding to the cellular receptor for LCMV, and GP-2 contains the fusion peptide and the transmembrane region (30).

Alpha-dystroglycan has been identified as a cellular receptor for LCMV (6). Dystroglycan is a dystrophin-associated glycoprotein, which connects the cytoskeleton with the extracellular matrix and is widely expressed in most tissues. It is posttranslationally cleaved into the highly glycosylated peripheral membrane protein alpha-dystroglycan, which is associated with the second product, the membrane-spanning protein beta-dystro-

glycan (12). However, some LCMV variants inefficiently interact with alpha-dystroglycan and also infect dystroglycan-negative cells. Thus, at least one alternative, yet-unidentified receptor or coreceptor for LCMV might exist (27, 29).

Here, we established efficient recombinant packaging systems for oncoretroviral and lentiviral pseudotypes with LCMV-GP. We analyzed the stability and constitutive production of LCMV-GP pseudotyped vectors as well as the transduction efficiency of various target cells including human primary cells. The results reveal the unique characteristics of the LCMV glycoproteins and make them an attractive alternative for pseudotyping of oncoretroviral and lentiviral vectors. In addition, we discuss the potential of the system for the functional characterization of LCMV-GP.

(This article is based on a study by W. R. Beyer performed in partial fulfillment of the requirements for a Ph.D. from the Faculty of Biology, University of Hamburg, Hamburg, Germany.)

MATERIALS AND METHODS

Cell lines and cell culture. ΦNXgp (293T cells constitutively expressing Mo-MLVgagpol) were a gift of G. P. Nolan. TELCeB (TE671 cells expressing Mo-MLVgagpol and the retroviral vector MFGnlsLacZ [9]) were kindly provided by F.-L. Cosset. 293T (human kidney epithelial), HT1080 (human fibroblast), HeLa (human cervical carcinoma), Cf2th (canine thymus), CHO (hamster ovary epithelial), and TE671 (human fibroblast) were obtained from the American Type Culture Collection. These cell lines were grown in Dulbecco's modified eagle medium (DMEM) with 1 mM pyruvate, 4 mM glutamine, and 10% heat-inactivated (30 min, 56°C) fetal bovine serum (DMEM/FBS). HUH7 (human hepatoma) were maintained in DMEM/FBS with 1× nonessential amino acids (Gibco-Invitrogen, Carlsbad, Calif.). SH-SY (human neuroblastoma), all NCE cell lines (human glioma [33]), and SC1 (mouse fibroblast) were grown in minimum essential medium eagle (MEM) with 1 mM pyruvate, 4 mM glutamine, and 10% FBS. K562 (human myeloid progenitor) was grown in RPMI 1640 (Gibco-Invitrogen) with 1 mM pyruvate, 4 mM glutamine, and 10% FBS (RPMI/FBS). TF1 (human myeloid progenitor) was grown in RPMI/FBS with 5×10^2 U of granulocyte-macrophage colony-stimulating factor/ml.

Construction of envelope protein expression plasmids. All envelope glycoprotein cDNAs were expressed by using the human cytomegalovirus immediate-early promoter, the rabbit beta-globin intron B, and rabbit beta-globin polyadenylation sites of the pHCMV vector (34). The coding regions of the originally published cDNA of LCMV-GP(WE) and the LCMV-GP(WE-HPI) variant, which we recently recloned from LCMV-infected cells, were inserted between the *Bam*HI sites of pHCMV as previously described (2). To generate comparable expression plasmids, which differ only in the coding regions, VSV-G (strain Indiana) cDNA and A-MLVenv (strain 4070A) cDNA were amplified by PCR (VSV-G primers, 5′ CTGGATCCGC CATGAAGTGC CTTTTGTACT TAG 3′ and 5′ CGGGATCCTT ATCACTTTCC AAGTCGGTTC ATC 3′; A-MLVenv-primers, 5′ ACGGATCCGC CATGGCGCGT TCAACGCTCT CA 3′ and 5′ TCGGATCCTT ATCATGGCTC GTACTCTATG G 3′). The PCR products were inserted between the *Bam*HI sites of pHCMV by using the restriction sites introduced by the PCR primers. The sequences of the inserted fragments in pHCMV-VSV-G, pHCMV-A-MLVenv, and the pHCMV-LCMV-GP expression plasmids were confirmed by DNA sequencing.

Transient production of retrovirus and lentivirus vector pseudotypes. Retroviral vectors were produced by transient transfection of 293T or ΦNXgp cells (22). Volumes of 5×10^6 cells were seeded in 10-cm-diameter culture dishes 16 h prior to transfection in DMEM/FBS. The culture medium was changed 1 h prior to transfection of 10 ml of DMEM/FBS/PS per dish with 25 μM chlororquine, 50 U of penicillin/ml, and 50 μg of streptomycin (PS; Gibco-Invitrogen)/ml. Then, 5 μg of pMP71-eGFP-pre (25), 10 μg of pSV-Mo-MLVgagpol, and 1 to 5 μg of a pHCMV envelope glycoprotein expression plasmid were used for the transfection of one culture dish. Vector production by ΦNXgp cells was also possible without the addition of pSVgagpol, as ΦNXgp cells already stably express Gag and Pol. However, titers were higher when the gagpol expression plasmid was included. A mixture containing 450 μl of the plasmids in ddH_2O and 50 μl of 2.5 M $CaCl_2$ was mixed well and then added dropwise to 500 μl of 2× HEPES-buffered saline (280 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1). After vortexing, the precipitate was immediately added to the cultures. The medium was changed after 8 h to 10 ml of DMEM/FBS/PS per dish with 20 mM HEPES. Twenty-three hours after transfection, the medium was changed to 7 ml of DMEM/FBS/PS per dish with 20 mM HEPES every 9 h to 15 h. The cell supernatants were filtered through a 0.22-μm-pore-size MILEX GP filter (Millipore, Bedford, Mass.) and used for analysis of vector pseudotypes. Lentivirus vectors were generated by transfecting 293T cells with 10 μg of pRRL.sinCMVeGFPpre, 5 μg of pRSV-Rev, 10 μg of pMDLg/pRRE (described in reference 11), and 1 to 7 μg of a pHCMV envelope glycoprotein expression plasmid.

Vector titration. Retroviral and lentiviral vector titers were measured by the transduction of various cell lines. Serial dilutions of the cell supernatants were prepared and 0.5 ml of each dilution was added to 5×10^4 cells, seeded in a well of a 24-well plate 4 h before transduction. Plates were centrifuged for 1 h at $1{,}000 \times g$. For vectors carrying the β-galactosidase marker gene, cells were fixed 65 h posttransduction in phosphate-buffered saline with 2% formaldehyde for 5 min, washed twice with phosphate-buffered saline, and incubated with staining solution (5 mM potassium ferricyanide, 5 mM ferrocyanide, 2 mM $MgCl_2$, and 80 μg of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside]/ml) for 24 h at 37°C. Titers were determined by counting blue colonies in each well under light microscopy and multiplying the result by the dilution factor.

For vectors carrying the enhanced green fluorescent protein (eGFP) marker gene, cells were analyzed 65 h posttransduction by flow cytometry on a FACS-Calibur (Becton Dickinson, San Jose, Calif.). Titers were calculated from dilutions, which resulted in 0.5 to 20% eGFP-positive cells, a range of linear relation between vector input and percentage of transduced cells, because multiple vector integrations into the target cell are generally not expected.

Generation of retroviral packaging cell lines constitutively expressing LCMV-GP. The envelope glycoprotein-negative cell line TELCeB (9), which constitutively expresses Mo-MLVgagpol and the retroviral vector MFGnlsLacZ, was used to generate a stable packaging cell line constitutively producing LCMV-GP-pseudotyped retroviral vectors. Samples containing 5×10^6 TELCeB cells were electroporated in 500 μl of DMEM with 20 μg of pHCMV-LCMV-GP(WE-HPI) and 1 μg of pCDNA3.1zeo (400 V, 1 ms; EPI 2500; Fischer, Heidelberg, Germany). Selection with DMEM/FBS containing 100 μg of zeocin (Invitrogen, Carlsbad, Calif.)/ml began 48 h after transfection. Cell clones were isolated after 10 days of selection and were grown in DMEM/FBS. Six-well plates containing 10^5 cells were seeded after different passage numbers. Fifteen hours later, cell supernatants were analyzed for the production of retroviral vector pseudotypes on TE671 as described above. Vector-producing cells and mock-electroporated cells were analyzed for LCMV-GP cell surface expression by flow cytometry with the monoclonal antibody KL25 (4).

A marker rescue assay was used to detect potential contaminating replication-competent helpervirus. Parental TELCeB cells, containing MLVgagpol and a retroviral vector with a *lacZ* as a marker gene, were cultured for 4 weeks in medium that was half fresh DMEM/FBS and half 0.22-μm-pore-size-filtered cell supernatants from vector-producing cells. Once per week, 10^5 of these cells were seeded in wells of a six-well plate. After 15 h, cell supernatant were filtered and analyzed for rescued *lacZ* retroviral vector genome on TE671 cells.

Analysis of vector stability. The stability of vector pseudotypes after freeze-thaw cycles was determined by freezing 0.5-ml aliquots of cell supernatants from transfected 293T cells at −70°C for 1 h and by thawing for 15 min at room temperature. Subsequently, the vector titers of the aliquots were determined on TE671 cells. The half-life of the vector pseudotypes was determined by incubating cell supernatants from transfected 293T cells at different temperatures. Aliquots were frozen after different time points at −70°C and finally titrated on TE671 cells. Alternatively, aliquots were first frozen at −70°C and subsequently incubated for different times in 24-well plates before the seeding of the target cells. Vector particles were concentrated from cell supernatants of transiently transfected cells by ultracentrifugation at 25,000 rpm and 4°C for 2 h by using an SW28 rotor. Supernatants were discarded after centrifugation, and the pellet was resuspended for 24 h at 4°C in DMEM.

Cell isolation and transduction of human primary glioma cells. Representative portions of freshly removed gliomas were first minced with scissors and then dispersed by rapid trituration in Hanks' balanced salt solution without calcium and magnesium. The undispersed fibrous material was allowed to settle. Single cells and small aggregates of the supernatant were centrifuged at $500 \times g$ for 5 min and resuspended in MEM/FBS. After further trituration, cells were directly plated in 24-well plates coated with an extracellular matrix derived from bovine corneal endothelial cells (32). Primary cells were maintained without subculture. Three to five days after plating, cells were transduced with retroviral or lentiviral pseudotyped vectors carrying the eGFP marker gene. Three days after transduction, cells were fixed in 95% ethanol–5% acetic acid and immunostained with monoclonal mouse-anti eGFP antibody (JL-8; Clontech, Palo Alto, Calif.) and polyclonal rabbit-anti-GFAP antibody (DAKO, Copenhagen, Denmark). The

TABLE 1. Production of LCMV-GP-pseudotyped oncoretroviral and lentiviral vectors

Transfected plasmids	Pseudotype titer [TU/ml] (± SD)[a]
RV + MLVgagpol + LCMV GP	1.3 (± 1.1) × 10^5
RV + LCMV GP	<10^2[b]
RV + MLVgagpol	<10^2
RV	<10^2
LV + HIVgagpol + HIVrev + LCMV GP	2.8 (± 2.3) × 10^5
LV + LVgagpol$^+$ HIVrev + LCMV GP	<10^2
LV + LVgagpol + LCMV GP	<10^2
LV + LCMV GP	<10^2
LV	<10^2

[a] Different combinations of LCMV-GP- and MLV-based oncoretroviral (RV) or HIV-based lentiviral (LV) packaging plasmids were cotransfected into 293T cells. Cell supernatants were analyzed by end-point dilutions on TE671 cells. Results are the mean vector titers and standard deviations of three experiments.
[b] Vector titer was lower than the detection limit.

relative numbers of transduced (eGFP positive) and untransduced (eGFP negative) glioma cells (GFAP positive) were determined by fluorescence microscopy and the counting of 10 camera fields for each pseudotype and glioma preparation. Tumors were obtained from a 51-year-old male (glioblastoma, WHO grade 4), a 61-year-old female (glioblastoma, WHO grade 4), and a 63-year-old male (oligodendroglioma, WHO grade 3).

Nucleotide sequence accession numbers. The DNA sequences of the expression plasmids pHCMV-LCMV-GP(WE), pHCMV-LCMV-GP(WE-HPI), pHCMV-VSV-G, and pHCMV-A-MLVenv have been submitted to the EMBL database under the accession numbers AJ318512, AJ318513, AJ318514, and AJ318515.

RESULTS

LCMV-GP pseudotypes oncoretroviral and lentiviral vectors. In order to determine whether recombinant LCMV glycoproteins can pseudotype oncoretroviral vectors, 293T cells were transfected with plasmids encoding LCMV-GP(WE-HPI), MLVgagpol, and a retroviral vector encoding eGFP. Cell surface expression of LCMV-GP in transfected 293T cells was shown by flow cytometry, and the incorporation of C-terminal hemagglutinin (HA)-tagged LCMV-GP into retroviral particles was detected by Western blot analysis of concentrated supernatants (data not shown). Infectious LCMV-GP pseudotyped retroviral vector was present in the supernatant of the transfected cells and were produced with >10^5 eGFP transfer units (TU) per ml. Plasmids expressing the vector genome, a gag/pol packaging plasmid, and the LCMV-GP were necessary and sufficient for the production of infectious pseudotyped retrovirus (Table 1).

Comparison of different LCMV-GP variants showed extreme variations in their ability to pseudotype retroviral vectors. The LCMV-GP(WE-HPI) cDNA, which we recloned from LCMV-infected cells (2) and which is efficiently processed and transported to the cell surface, pseudotyped retroviral vectors with high efficiency, leading to titers of >10^5 TU/ml. In contrast, the glycoprotein encoded by the originally published LCMV-GP(WE) cDNA, which, as we described before, is not processed and expressed on the cell surface, did not pseudotype retroviral vectors. In a previous study (2), we showed that the lack of surface expression was associated with a proline at position 110 in the original LCMV-GP(WE) cDNA instead of a leucine found in the newly cloned LCMV-GP(WE-HPI). Accordingly, analysis of artificial cDNA variants with single missense mutations showed that LCMV-GP (WE-HPI) with a leucine$_{110}$-to-proline$_{110}$ mutation impedes, while LCMV-GP(WE) with a proline$_{110}$-to-leucine$_{110}$ mutation allows the generation of infectious particles (Table 2). In conclusion, only correct surface expression of GP was associated with the generation of infectious pseudotypes.

Gene transfer vectors derived from lentiviruses are an alternative to the originally developed and widely used oncoretroviral vectors. Therefore, we analyzed whether LCMV-GP could also mediate the infectivity of human immunodeficiency virus (HIV)-based lentiviral vectors. 293T cells were transfected with four packaging plasmids encoding the lentiviral vector genome with eGFP as a marker gene, HIVgagpol, HIVrev, and LCMV-GP. The cell supernatants were analyzed for infectious lentiviral particles as measured by transfer of the eGFP marker gene. LCMV-GP was found to pseudotype HIV-based lentiviral vectors very well and all four packaging plasmids were required for pseudotyping (Table 1).

The efficiency of pseudotype formation with oncoretroviral and lentiviral vectors was analyzed for LCMV-GP in comparison with the widely used envelope protein VSV-G and the retroviral A-MLVenv. Coding sequences of the three glycoproteins were cloned into the same position of the pHCMV vector. These comparable expression plasmids were used to package the three pseudotypes in parallel under equivalent conditions with plasmid concentrations within the optimal range for all three envelope glycoproteins, and supernatants were then assayed for infectious vector particles. Transient transfections resulted in similar vector titers for all three pseudotypes. However, for oncoretroviral vectors and for lentiviral vectors, A-MLVenv and VSV-G, respectively, tended to give rise to slightly higher titers than the other envelope proteins (Table 3). Comparable results were obtained after titration on HeLa, HT1080, TE671, and 293T cells (Table 4). Thus, LCMV-GP pseudotypes oncoretroviral and lentiviral vectors with an efficiency similar to those of the widely used envelope proteins A-MLVenv and VSV-G.

Pseudotypes with LCMV-GP have high stability and can be concentrated by ultracentrifugation without loss of vector titer. In contrast to VSV-G pseudotypes, amphotropic retroviruses lose their infectivity after concentration by ultracentrifugation. Here, LCMV-GP-pseudotyped retroviral vectors were concentrated by ultracentrifugation and the stability was compared with VSV-G- or A-MLVenv-pseudotyped particles. The

TABLE 2. Proline-to-leucine mutation at amino acid 110 leads to generation of infectious LCMV-GP-pseudotyped vectors

LCMV-GP variant	Pseudotype titer [TU/ml] (± SD)[a]
Control	<10^2[b]
LCMV-GP(WE-HPI)	6.1 (± 5.2) × 10^5
LCMV-GP(WE)	<10^2
LCMV-GP(WE_{P110L})	2.4 (± 2.1) × 10^5
LCMV-GP($WE\text{-}HPI_{L110P}$)	<10^2

[a] Expression plasmids for different LCMV-GP variants or an empty plasmid (control) were contransfected with oncoretroviral packaging plasmids into 293T cells. Cell supernatants were analyzed by end-point dilutions on TE671 cells. Results are the mean vector titers and standard deviations of three experiments.
[b] Vector titer was lower than the detection limit.

TABLE 3. LCMV-GP-pseudotyped oncoretroviral and lentiviral vectors

Vector type	Mean pseudotype titer [TU/ml] (± SD)[a]		
	LCMV-GP	VSV-G	A-MLVenv
RV	$4.0 (\pm 2.8) \times 10^5$	$2.5 (\pm 2) \times 10^5$	$12 (\pm 9) \times 10^5$
LV	$4.4 (\pm 3.7) \times 10^5$	$17 (\pm 4.1) \times 10^5$	$4.1 (\pm 1.9) \times 10^5$

[a] Viral envelope proteins LCMV-GP(WE-HPI), VSV-G, or A-ML Venv were coexpressed with MLV-based oncoretroviral (RV) or HIV-based lentiviral (LV) packaging plasmids. Titers of oncoretroviral pseudotypes were determined on TE671 cells, and titers of lentiviral pseudotypes were determined on 293T cells. Results are the mean vector titers and standard deviations of at least three independent experiments.

TABLE 5. LCMV-GP-pseudotyped vectors can be concentrated by ultracentrifugation

Pseudotype glycoprotein	% Recovery after concentration[a] (± SD)
LCMV-GP	103 (± 62)
VSV-G	94 (± 55)
A-MLVenv	0.3 (± 0.2)

[a] The vector titer of oncoretroviral pseudotypes was determined before and after concentration by ultracentrifugation. The initial pseudotype titers were 5×10^4 to 2×10^6 TU/ml. The volume before concentration was 37 ml and was 0.5 ml to 1 ml after concentration. Results are the means and the standard deviations of the calculated recovery from three independent experiments.

volumes and vector titers were determined for all three pseudotypes before and after concentration. LCMV-GP- and VSV-G-pseudotyped vectors showed similar stability and total recovery of vector titer. In contrast to LCMV-GP pseudotyped particles, dramatic loss of infectivity was observed for A-MLVenv pseudotypes (Table 5).

To study the stability of the retroviral pseudotypes, the infectivities of vector supernatants were determined after different incubation periods at 37, 23, and 4°C. Transducing units of retroviral pseudotypes with LCMV-GP showed an average half-life of 7 h at 37°C, 38 h at 23°C, and 11 days at 4°C. These results are similar to the stability of retroviral pseudotypes with VSV-G (7 h, 44 h, 10 days) or A-MLVenv (7 h, 30 h, 6 days), which were analyzed in parallel. After freezing at −70°C and thawing at room temperature, LCMV-GP pseudotypes showed a less than 50% loss of virus titer after four freeze-thaw cycles and were at least as stable as the A-MLVenv- and VSV-G-containing particles, as determined in parallel (data not shown).

Generation of retroviral packaging cell lines constitutively expressing LCMV-GP. VSV-G has been successfully used to generate retroviral pseudotypes, but its use is limited due to cell toxicity. Thus, it has not been possible to generate packaging cell lines constitutively expressing VSV-G. To test whether packaging cell lines expressing LCMV-GP constitutively could be generated, the retroviral packaging cell line TELCeB was cotransfected with the expression plasmid for LCMV-GP and plasmids conferring antibiotic resistance. TELCeB cells express MLVgagpol and have a retroviral vector genome carrying the LacZ marker gene but lack an envelope glycoprotein gene. After selection, individual clones of the LCMV-GP-transfected cells were analyzed. Several clones of independent experiments produced significant titers of LacZ TU. The titer of the LCMV-GP expressing TELCeB-GP clones remained stable during cultivation and after freezing and thawing of the cells. An example of one clone is shown in Fig 1. To test for contamination with helper virus, TELCeB cells were cultivated for 4 weeks with supernatants from TELCeB-GP clones. No retrovirus transferring the LacZ marker gene was detected during this period in the supernatant of the indicator cells, which excludes the presence of a contaminating helper virus (Fig. 1A). Furthermore, LCMV-GP expression could be detected on the cell surface of stable packaging cell lines by flow cytometry (Fig. 1B). This shows that unlike VSV-G pseudotypes, LCMV-GP-pseudotyped vectors can be produced by stable retroviral packaging cell lines.

TABLE 4. Relative vector titers of pseudoptyped oncoretroviral vectors on different cell lines

Cell line (no. of expt)	Relative pseudotype titer to TE671 (± SD)[a]		
	LCMV-GP	VSV-G	A-MLVenv
TE671	1	1	1
293T (4)	0.5 (± 0.4)	0.4 (± 0.1)	0.7 (± 0.3)
HT1080 (2)	2.1 (± 0.3)	0.7 (± 0.1)	1.1 (± 0.2)
HeLa (4)	0.8 (± 0.6)	2.0 (± 0.8)	0.8 (± 0.3)

[a] Viral envelope proteins LCMV-GP(WE-HPI), VSV-G, or A-MLVenv were coexpressed with MLV-based oncoretroviral packaging plasmids in 293T cells. Cell supernatants were analyzed by end-point dilutions on different cell lines. Results are the means and standard deviations of the relative vector titers to the titer on TE671 cells and of at least two independent experiments.

LCMV-GP pseudotypes transduce many human cell types and cells from different species. Wild-type LCMV has been shown to infect several cell types from different tissues. To analyze the host range of LCMV-GP retroviral pseudotypes, we determined the vector titer on various cells by end-point dilutions. Cell lines from different species, such as human, mouse, hamster, and dog, were efficiently transduced. In addition to fibroblast and epithelial cell lines, LCMV-GP also mediated entry into TF1 and K562 hematopoetic cell lines, the hepatoma cell line HUH-7, the neuroblastoma SH-SY, and the glioma cell line NCE-G112 (Table 6). Thus, LCMV-GP mediates gene transfer into various cell lines from different human cell types, which are relevant targets for gene therapy.

LCMV-GP pseudotypes transduce primary human glioma cells. Tumors derived from the central nervous system (CNS), like malignant gliomas, have a very poor prognosis. These cells have been a primary target for experimental cancer gene therapy and were analyzed in detail as one of many possible target cells for LCMV-GP-pseudotyped vectors. Initially, we performed endpoint dilutions of different retroviral pseudotypes on various human glioma cells derived from 4- to 64-year-old patients with astrocytomas or glioblastomas. In contrast to A-MLVenv retroviral pseudotypes, LCMV-GP pseudotypes efficiently transduced all kinds of established human glioma cell lines (Fig. 2). Human glioma cells were also transduced in primary cultures derived from fresh surgical specimens. These primary human glia cells from an oligodendroglioma and two glioblastomas could be efficiently transduced by LCMV-GP retroviral pseudotypes as shown by eGFP marker gene expression of GFAP-positive glia cells (Fig. 3). The different intensities of fluorescence most likely reflect the heterogeneity of the cell culture. Semiquantitative analysis of eGFP and GFAP colocalizations revealed that transduction efficiencies for

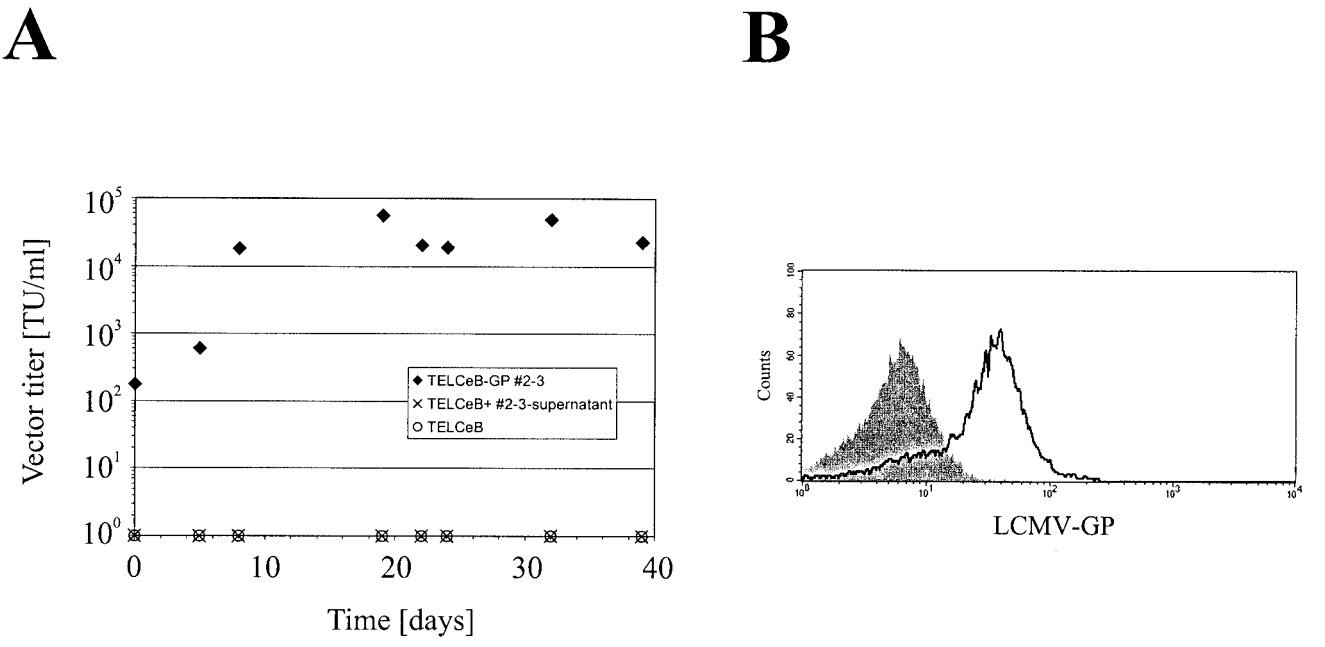

FIG. 1. Stable retroviral packaging cell lines that constitutively produce LCMV-GP-pseudotyped retroviruses. (A) The envelope protein-negative packaging cell line TELCeB was transfected with LCMV-GP and a zeocin resistance expression plasmid. After selection with zeocin, the pseudotype titers of clone TELCeB-GP #2-3 were analyzed for several weeks of cell culture by end-point dilutions on TE671, the parental cell line of TELCeB. Results are shown as TU of the *lacZ* marker gene per milliliter, which was carried by the retroviral vector. A cell clone from mock-transfected cells (TELCeB) as well as parental TELCeB cells, which were cocultivated with clone #2-3 supernatants (TELCeB+ #2-3 supernatants), were analyzed in parallel. (B) Fluorescence-activated cell sorter analysis of TELCeB-GP #2-3. Mock-transfected cells (shaded lines) or TELCeB-GP #2-3 cells (open lines) were analyzed by flow cytometry with the LCMV-GP-directed monoclonal antibody KL25.

LCMV-GP pseudotyped vectors are at least as efficient as those of A-MLVenv pseudotypes (Table 7).

DISCUSSION

Oncoretroviral and lentiviral vectors can be pseudotyped with glycoproteins of various origins. However, application of these pseudotypes is generally limited by one or more factors, such as low vector titers, instability of infectivity, limited host range, or toxicity of the glycoproteins. Here, we have demonstrated that with regard to these characteristics, LCMV-GP is an attractive alternative to currently used envelope proteins for pseudotyping oncoretroviral and lentiviral vectors.

We have shown that the LCMV-GP are incorporated onto the surface of retroviral and lentiviral vectors and mediate envelope glycoprotein function in the process of viral entry

TABLE 6. Host range of LCMV-GP-pseudotyped retroviral vectors

Cell line	Cell type	Pseudotype titer[a] [TU/ml] (± SD)
Te671	Fibroblast, human	$6.9 (\pm 2.0) \times 10^5$
HeLa	Epithelial, cervix, human	$5.1 (\pm 2.4) \times 10^5$
293	Epithelial, kidney, human	$9.4 (\pm 5.8) \times 10^5$
nce-G112	Glioma, human	$1.2 (\pm 5.9) \times 10^6$
SH-SY	Neuroblastoma, human	$3.4 (\pm 1.3) \times 10^5$
TF1	Myeloid progenitor, human	$1.4 (\pm 8.9) \times 10^4$
K562	Myeloid progenitor, human	$1.8 (\pm 9.0) \times 10^5$
HUH7	Hepatoma, human	$3.5 (\pm 2.5) \times 10^4$
CHO	Epithelial, ovary, hamster	$7.6 (\pm 6.1) \times 10^4$
Cf2th	Thymus, canine	$2.8 (\pm 2.2) \times 10^6$
SC1	Fibroblast, embryo, mouse	$1.8 (\pm 4.2) \times 10^6$

[a] Target cells were transduced with serial dilutions of LCMV-GP-pseudotyped oncoretroviral vector. Results are the mean vector titers and the standard deviations from three end-point dilutions.

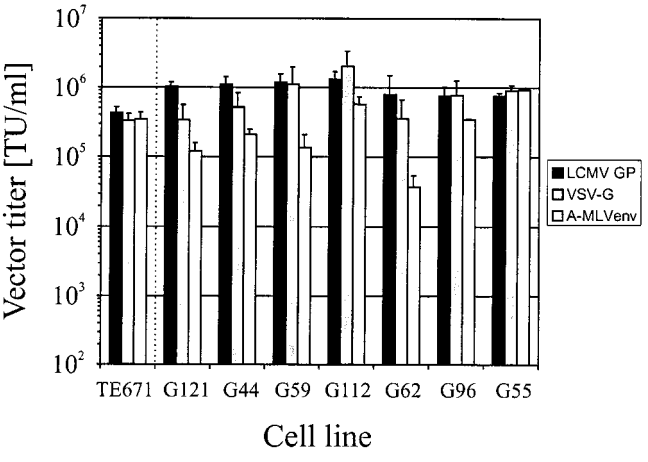

FIG. 2. LCMV-GP-pseudotyped vectors are superior to A-MLVenv particles for transduction of human glioma cell lines. Target cells were transduced with serial dilutions of LCMV-GP-, VSV-G-, or A-MLV-env-pseudotyped oncoretroviral vectors encoding eGFP. Transduced, eGFP-positive cells were quantified by flow cytometry. Results are the mean vector titers and standard deviations from three end-point dilutions.

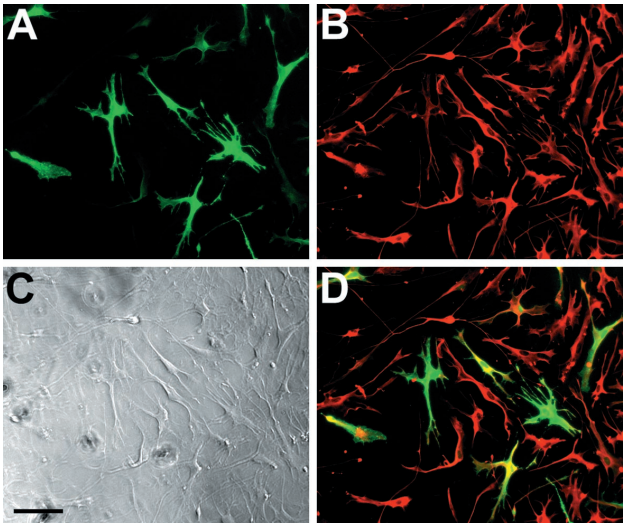

FIG. 3. LCMV-GP-pseudotyped vectors transduce primary human glioma cells. Cells from a primary human glioblastoma were resuspended and directly plated on coverslips. The cells we transduced 4 days after plating with LCMV-GP-pseudotyped lentiviral vectors encoding eGFP. Three days after transduction, the cells were analyzed by immunostaining with monoclonal anti-eGFP antibody (A) and polyclonal anti-GFAP antibody (B). Shown are the phase contrast (C) and the eGFP/GFAP overlay (D). Bar, 100 μm.

without requiring other LCMV proteins. The production of recombinant vector particles with LCMV-GP was possible only after we had established a system for recombinant cell surface expression of processed LCMV-GP (2). We found that functional expression of the originally published LCMV-GP(WE) cDNA is blocked by a single missense mutation encoding a proline at amino acid 110. In contrast, the LCMV-GP(WE-HPI) variant, which we recloned from LCMV-infected cells, encoded various different amino acids, including a leucine at amino acid 110, and efficiently pseudotyped retroviral vectors. Mutational analysis of these two LCMV-GP variants showed that the proline at amino acid 110 impedes while a leucine at amino acid 110 allows functional expression as shown by the infectivity of the generated retrovirus vector particles. Thus, functional high-level expression of LCMV-GP was achieved, independent of other LCMV proteins, after correction of a single missense mutation in the original cDNA clone.

Recombinant oncoretroviral and lentiviral vector particles with LCMV-GP were compared with the widely used A-MLVenv- and VSV-G-pseudotyped vectors. Vector titers after transient production were in the same order of magnitude for all three pseudotypes. However, 50 h after transient transfection of 293T cells, production of VSV-G-pseudotyped vectors decreased constantly and much more dramatically than for A-MLVenv- or LCMV-GP-pseudotyped vectors. This effect was even more pronounced for VSV-G pseudotypes when more than the usual 2-μg glycoprotein expression plasmids were used and thus might be a result of VSV-G cytotoxicity (data not shown). The significant toxicity may also be the reason that we and other groups were not successful in generating packaging cell lines constitutively expressing VSV-G. However, the generation of stable packaging cell lines is an important step for well-defined and safe production of vector particles. Cell lines in which VSV-G is regulated by tetracycline have been established, and these cells are useful for the manufacturing of VSV-G-pseudotyped particles. However, after induction of VSV-G, pseudotypes are produced for only a limited period from already dying packaging cells (7). In this study, we established retroviral packaging cell lines constitutively expressing LCMV-GP and therefore constantly producing LCMV-GP-pseudotyped vectors. The maximum expression levels of LCMV-GP, which are tolerated by different packaging cell lines, still need to be determined. However, stable and constitutive expression of LCMV-GP sufficient for packaging retroviral vectors was found to be possible.

Applications of retroviral and lentiviral vectors, especially in vivo, are limited due to relatively low vector titers generated after stable or transient production. Therefore, it is often essential to concentrate the particles, which can easily be accomplished by high-speed centrifugation of the cell supernatants. However, retrovirus vector particles cannot be concentrated by ultracentrifugation because of the instability of the retroviral envelope. In contrast, we could concentrate LCMV-GP-pseudotyped retroviral vectors without loss of infectivity. Similar stability was previously reported for VSV-G-pseudotyped vectors (5). To further characterize pseudotype stability, the infectivities of vector supernatants were determined for the

TABLE 7. LCMV-GP-pseudotyped vectors transduce primary human glioma cells[a]

Target cells	Lentiviral pseudotypes				Oncoretroviral pseudotypes			
	Vector pseudotype (TU on 293T cells)	Transduced glioma cells[b]	Glioma cells[c]	Ratio (%)[d]	Vector pseudotype (TU on 293T cells)	Transduced glioma cells[b]	Glioma cells[c]	Ratio (%)[d]
Glioblastoma	LCMV-GP (10^3)	3.6	10.6	34	LCMV-GP (5×10^3)	0.3	5.5	5
	A-MLVenv (10^3)	0.9	7	13	A-MLVenv (6×10^3)	0.2	4.8	4
	VSV-G (5×10^3)	5.2	7.6	68	VSV-G (3×10^3)	0.7	8.9	8
Oligodendroglioma	LCMV-GP (10^3)	2.8	69	4	LCMV-GP (5×10^3)	1	55	2
	A-MLVenv (10^3)	0.7	38	2	A-MLVenv (6×10^3)	0.4	38	1
	VSV-G (5×10^3)	13	64	20	VSV-G (3×10^3)	0.4	64	0.6

[a] Human primary glioblastoma or oligodendroglioma cells were transduced with 1×10^3 to 6×10^3 eGFP TU of LCMV-GP-, A-MLVenv-, or VSV-G-pseudotyped oncoretroviral or lentiviral vectors. Cells were analyzed by fluorescence microscopy after immunostaining with monoclonal anti-eGFP antibody and polyclonal anti-GFAP antibody (glia antigen). Results are the mean cell numbers from 10 random camera fields.
[b] Mean cell numbers of $eGFP^+$ $GFAP^+$ double-positive transduced glioma cells from 10 random camera fields.
[c] Mean cell numbers of $GFAP^+$ glioma cells from 10 random camera fields.
[d] Percent $eGFP^+$ $GFAP^+$ double-positive cells within the total number of $GFAP^+$ glioma cells.

pseudotypes after different incubation periods at 4, 23, or 37°C, as well as after multiple freeze-thaw cycles. The LCMV-GP pseudotypes were found to be at least as stable as A-MLVenv- and VSV-G-pseudotyped vectors, which is an important feature for several gene therapeutic applications.

Several cell types from various tissues have been shown to be susceptible to infection with wild-type LCMV (14, 31). Furthermore, alpha-dystroglycan has been identified as a cellular receptor of LCMV and was found to be expressed in most tissues (6, 12). Correspondingly, LCMV-GP-pseudotyped vectors efficiently transduced various cell lines from different tissues, relevant for gene therapy.

Transduction of human glioma cells as one of many possible targets was studied in further detail. Malignant glioma have been a major target for cancer gene therapy because of the poor prognosis of the affected patients (24). These brain tumors have retained their dismal prognosis despite advances in neurosurgery, radiation, and chemotherapy, but different gene therapeutic approaches offer the promise of augmenting traditional cancer therapies (1, 17). The therapeutic genes on the vectors can act to directly kill or block the growth of the tumors cells, to inhibit angiogenesis, or to stimulate immune responses to the tumor.

Oncoretrovirus-based vectors have been the most widely used system for clinical gene therapy protocols. However, these vectors only transduce dividing cells, and within a short treatment window, most tumor cells are not dividing. This limited cell cycling may explain why gene transfer using oncoretroviral vectors was found to be less efficient in this study than for lentiviral vectors (Table 6). Accordingly, efficient gene transfer into tumors in vivo was difficult to achieve, although transduction efficiency was improved by the grafting of retroviral packaging cell lines (10, 28). Conversion of tumor cells to vector-producing cells may be an alternative to further enhance gene transfer efficiency (15, 26). The clinical trials showed that the use of retroviral vectors in the CNS is relatively safe, but the therapeutic effect was low or absent, most likely due to inefficient gene delivery (24). In this study, we achieved efficient gene transfer of LCMV-GP-pseudotyped vectors into human oligodendroglioma, astrocytoma, and glioblastoma cells. Moreover, pseudotypes with LCMV-GP were found to be more efficient than amphotropic retroviral pseudotypes for ex vivo gene transfer into several human glioma cell lines and at least as efficient as MLV-A for transfer into other glioma lines and into primary human glioma cells.

In this study, we showed that LCMV-GP-pseudotyped vectors can be concentrated by ultracentrifugation and efficiently transduced human glioma cells. These features make LCMV-GP pseudotypes an interesting alternative to VSV-G for direct vector application into the CNS, for which very small volumes of highly concentrated vector are used. In previous studies in vivo, the vast majority of transduced cells were differentiated neurons after injection of concentrated, VSV-G-pseudotyped lentiviral vectors into brain regions like the striatum and substantia nigra (3, 16). However, in applications such as Parkinson's disease, the wide distribution of proteins expressed by neuronal cells in the CNS by their axons can be a disadvantage over the more confined expression from glia cells. Therefore, it would be interesting to know whether LCMV-GP pseudotypes efficiently transduce glia cells also in vivo. Gene transfer into different target cells of the CNS in vivo still has to be analyzed in further studies. However, the broad host range already make LCMV-GP-pseudotyped vectors an attractive alternative for gene transfer into many cell types.

In addition to the use as a promising gene therapy tool, retroviral pseudotypes with LCMV-GP also provide the unique possibility for functional characterization of LCMV-GP variants without the need to establish reverse genetics for LCMV. With recombinant pseudotypes, the capacity of a particular glycoprotein variant to promote viral entry can be directly analyzed. Of special interest are antibody or cytotoxic T-lymphocyte escape variants. The recombinant LCMV-GP-pseudotyped vectors can also be used as a tool to analyze the function and host range of known, as well as newly generated, glycoprotein variants independently of other LCMV proteins. Single-amino-acid changes in the LCMV glycoprotein have been described to alter LCMV tropism. Furthermore, as some LCMV variants inefficiently interact with alpha-dystroglycan and infect dystroglycan-negative cells, there might exist at least one alternative, yet-unidentified LCMV receptor or coreceptor (27, 29). LCMV-GP tropism variants could also be used to direct gene transfer vectors to a given cell type and thereby modify the host range.

In conclusion, although the potential of LCMV-GP-pseudotyped oncoretroviral and lentiviral vectors for human gene therapy remains to be analyzed in detail, the unique characteristics of these pseudotypes regarding stability, toxicity, and host range are highly attractive for gene transfer applications. Furthermore, the established system should allow functional characterization of various natural and artificial arenavirus glycoprotein variants.

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