



Receptor use by pathogenic arenaviruses

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Abstract

The arenavirus family contains several important human pathogens including Lassa fever virus (LASV), lymphocytic choriomeningitis virus (LCMV) and the New World clade B viruses Junin (JUNV) and Machupo (MACV). Previously, α -dystroglycan (α -DG) was identified as a receptor recognized by LASV and certain strains of LCMV. However, other studies have suggested that α -DG is probably not used by the clade B viruses, and the receptor(s) for these pathogens is currently unknown. Using pseudotyped retroviral vectors displaying arenavirus glycoproteins (GPs), we are able to explore the role played by the GP in viral entry in the absence of other viral proteins. By examining the ability of the vectors to transduce DG knockout murine embryonic stem (ES) cells, we have confirmed that LASV has an absolute requirement for α -DG in these cells. However, the LCMV GP can still direct substantial entry into murine ES cells in the absence of α -DG, even when the GP from the clone 13 variant is used that has previously been reported to be highly dependent on α -DG for entry. We also found that neither LASV or LCMV pseudotyped vectors were able to transduce human or murine lymphocytes, presumably due to the glycosylation state of α -DG in these cells. In contrast, the JUNV and MACV GPs displayed broad tropism on human, murine and avian cell types, including lymphocytes, and showed no requirement for α -DG in murine ES cells. These findings highlight the importance of molecules other than α -DG for arenavirus entry. An alternate receptor is present on murine ES cells that can be used by LCMV but not by LASV, and which is not available on human or murine lymphocytes, while a distinct and widely expressed receptor(s) is used by the clade B viruses.

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Introduction

The arenaviruses are rodent-borne pathogens which include five members classified by the Centers for Disease Control and Prevention as Category A biological agents, capable of posing a significant threat if used for bioterrorism purposes. Two major subgroups are recognized on the basis of sequence comparisons, the Old World and New World viruses, with the New World viruses further divided into clades A, B and C (Fig. 1). The most

common human infections are caused by the Old World viruses, lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LASV). While LCMV infections are usually asymptomatic or mild, LASV infection is characterized by pulmonary edema, respiratory distress, shock and bleeding from mucosal surfaces. LASV infection rates are estimated at over 300,000 per year, mostly in Western Africa. Serious illnesses are also associated with certain New World clade B viruses, including Junin (JUNV) and Machupo (MACV) viruses, the causative agents of Argentine and Bolivian hemorrhagic fevers, respectively. Since studies of the highly pathogenic arenaviruses are hampered by the need for biosafety level (BSL)-4 containment laboratories, most of the available information about this family has come from studies of LCMV.

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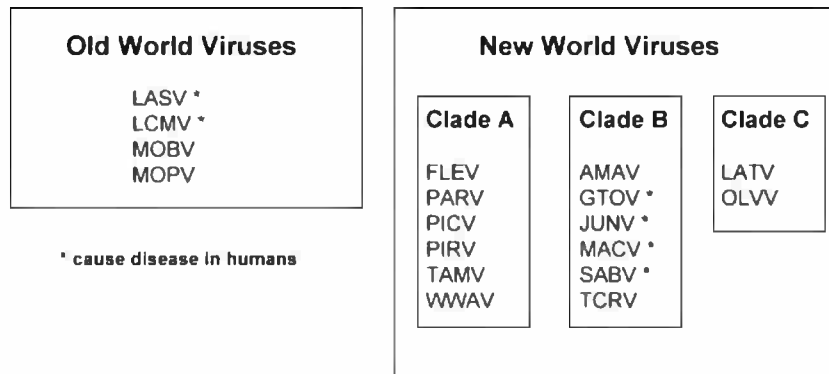


Fig. 1. The Arenaviridae. The family is divided into New World and Old World viruses, with the New World viruses further divided into three clades. The strains that are pathogenic for humans are indicated.

Arenaviruses are enveloped, bisegmented single-stranded RNA viruses. Their entry into cells is directed by a fusion glycoprotein (GP) located in the outer lipid envelope of the virus. This protein is initially synthesized as a precursor protein, GPC, which is cleaved into two non-covalently linked subunits, an integral membrane protein, GP2, and a peripheral membrane protein, GP1. The binding site for the cellular receptor is contained within GP1 (Parekh and Buchmeier, 1986), while GP2 anchors the complex in the viral and host cell membranes (Burns and Buchmeier, 1991) and contains structural features typical of class I viral fusion proteins (Gallagher et al., 2001). We and others have demonstrated that arenavirus GPs can be incorporated into retroviral and lentiviral vector particles, generating functional surrogates of the arenavirus entry process that can be worked with under standard BSL-2 laboratory conditions (Beyer et al., 2002; Christodoulopoulos and Cannon, 2001; Kunz et al., 2003). The use of pseudotyped retroviral vectors also simplifies the analysis of arenavirus entry as it is possible to study the function of GP in the absence of other factors that may influence infection by native viruses.

A cellular receptor for LASV, certain strains of LCMV and the New World clade C arenaviruses Oliveros and Latino has been identified as α -dystroglycan (α -DG) (Cao et al., 1998; Kunz et al., 2005; Spiropoulou et al., 2002). This is a peripheral membrane protein, non-covalently associated with β -DG, and widely expressed in most cells, where it serves as an anchor between the submembranous cytoskeleton and the extracellular matrix (Durbecj et al., 1998). Recently, it has been shown that the specific post-translational glycosylation of α -DG is necessary both for its interaction with the extracellular matrix (Michele and Campbell, 2003) and for its function as a receptor for LCMV (Imperiali et al., 2005; Kunz et al., 2005) and LASV (Kunz et al., 2005).

It is also clear that α -DG is not the only receptor to be used by this family of viruses. Several isolates of LCMV have been shown capable of infecting murine ES cells knocked-out for α -DG, for example, variants WE2.2 and PBL364 gave wild-type infection rates on the knockout cells (Smelt et al., 2001; Kunz et al., 2004). Similarly, representative clade A (Parana) and clade B (Amapari) New World viruses have been shown to infect

murine ES cells independently of α -DG (Spiropoulou et al., 2002).

In this report, we used pseudotyped retroviral vectors to examine cell tropism and receptor usage by both Old World and New World viruses and in particular to further investigate the requirement for α -DG. Our data demonstrate that α -DG plays no role in the entry of the clade B viruses, JUNV and MACV, and provide additional evidence that alternate, non- α -DG receptor molecules can be used by LCMV.

Results

Generation of retroviral vectors pseudotyped with arenavirus GPs

In order to examine GP–receptor interactions during arenavirus entry, we generated a panel of retroviral vector particles pseudotyped with the GPs from different pathogenic arenaviruses. The use of pseudotyped vectors allows to examine the arenavirus entry process in isolation from the rest of the virus life cycle and to study BSL-4 pathogens under standard BSL-2 laboratory conditions. The panel we used included the Old World viruses LASV and LCMV and the New World clade B viruses JUNV and MACV, together with the non-pathogenic clade B virus Tacaribe (TACV) (Fig. 1). For LCMV, we used GPs from both the prototypical Arm53b strain and a mutated version of the protein with the substitution F₂₆₀L at the C-terminus of GP1 (Arm-F₂₆₀L). This mutation corresponds to the single change present in the GP of clone 13, an immunosuppressive variant of Arm53b (Salvato et al., 1991) that is reported to have greater dependency on α -DG for entry (Smelt et al., 2001).

Pseudotyped retroviral vectors were generated as previously described (Christodoulopoulos and Cannon, 2001; Soneoka et al., 1995). The vectors expressed GFP reporter genes, allowing us to measure the efficiency of GP function by measuring transduction rates (titer) by FACS analysis of target cells incubated with the vector particles. All vector stocks were initially titered on human 293A cells, which are efficiently transduced by all of the different vectors used. The average titers obtained for unconcentrated vector stocks are shown in

Fig. 2. We consistently observed that the titers for the vectors pseudotyped with the Old World GPs were one order of magnitude higher than those obtained with the New World clade B GPs. In addition, in each experimental pair tested, the titer of the Arm-F₂₆₀L vectors was slightly higher than those obtained with the Arm53b vectors, although the difference was not statistically significant when averaged out over 5 data sets.

Incorporation of arenavirus GPs into retroviral vector particles

In order to examine whether the variation in titer on 293A cells between the different pseudotyped vectors arose because of differences in the efficiency of incorporation of GPs into vector particles, we performed Western blot analysis on purified vector particles. However, this analysis was complicated by the fact that we could not find a broadly cross-reacting antibody that recognized all of the arenavirus GPs used in this study. For example, monoclonal antibody WE-83.6, directed against the LCMV GP2 subunit, recognized only the LASV and LCMV GP2 proteins (data not shown), while the anti-LASV GP1 monoclonal antibody L52-161-6 recognized only the LASV protein (Fig. 3A). In contrast, the anti-JUNV monoclonal antibody QD04 AF03 (Sanchez et al., 1989) recognized proteins from both JUNV and LCMV, although it did not cross-react with MACV, TACV or LASV proteins (Fig. 3B and data not shown).

The proteins recognized by antibody QD04 AF03 for both the JUNV and LCMV pseudotyped vectors were approximately 65 kDa (JUNV only) and 33 kDa. The 65 kDa species is probably the uncleaved JUNV GPC precursor as this species was more prevalent in cell lysates than in viral particles (data not shown), but the identity of the 33 kDa protein was more difficult to ascertain. Previously, the LCMV GP1 subunit has been reported to run at 44 kDa and the GP2 subunit at 35 kDa (Burns and Buchmeier, 1991), suggesting that the antibody is recognizing GP2 subunits. However, QD04 AF03 has been reported to recognize the JUNV GP1 subunit (York et al., 2005), where the size of the JUNV GP1 and GP2 subunits was found to

be approximately 33 and 35 kDa respectively. To determine whether antibody QD04 AF03 was detecting the GP1 or GP2 subunits of JUNV and LCMV, we performed Western analysis using deglycosylated viral proteins and compared the sizes of the bands we saw to the predicted molecular weights for the JUNV and LCMV GP1 and GP2 subunits. This analysis revealed that the anti-JUNV antibody was probably recognizing the GP1 subunits of both viruses (Fig. 3B). The finding that the LCMV GP1 subunit runs at approximately 33 kDa in our system instead of the previously reported 44 kDa was unexpected. Possible explanations for this discrepancy include the fact that previous studies have used native viruses rather than pseudotyped vectors, and the different cell types used may result in different glycosylation patterns that affect the size of the mature cleaved GP1 subunit.

Interestingly, we detected a significant amount of unprocessed JUNV GPC in viral particles, which was not seen with the LCMV GP (Fig. 3B). This suggests that the lower titers obtained for the clade B vectors on 293A cells could result from less efficient processing of the GPC precursor, although it is also possible that this difference arises from the ability of the antibody to detect the JUNV but not the LCMV GPC precursor. Alternatively, the differences between the titers of the clade B and Old World pseudotypes could reflect the use of different cellular receptors on the 293A cell surface. We note that the one-log differences in titer that we observed between the clade B and Old World vectors on 293A cells did not hold up for every cell line we tested (see below).

As stated above, the LCMV Arm-F₂₆₀L variant always gave slightly higher titers on 293A cells than the parental Arm53b vectors, although this was not statistically significant (Fig. 2). The presence of leucine or isoleucine at position 260 is typical of the immunosuppressive strains of LCMV that cause persistent infections in mice (Sevilla et al., 2000) and which exhibit altered pathogenicity and tissue tropism in vivo (Ahmed and Oldstone, 1988; Ahmed et al., 1984; Sevilla et al., 2000; Smelt et al., 2001). Arm53b and clone 13 have been reported to show large differences in their in vitro affinities for α -DG, with clone 13 binding α -DG with 100–1000 times greater affinity than Arm53b in virus overlay protein binding assays (VOPBAs) (Sevilla et al., 2000; Smelt et al., 2001). In addition, clone 13 is significantly more dependent on the presence of α -DG to infect mouse ES cells (Kunz et al., 2004; Smelt et al., 2001).

To investigate the basis for the increase in titer resulting from the F₂₆₀L substitution, we examined whether the LCMV Arm-F₂₆₀L GP was incorporated at higher efficiency into retroviral vector particles. However, Western blot analysis revealed that both the GP1 and GP2 subunits of Arm-F₂₆₀L were incorporated into retroviral particles at significantly lower levels than the Arm53b protein. Furthermore, higher levels of Arm-F₂₆₀L GP1 were observed in the lysates of transfected cells when compared to Arm53b, which is consistent with a reduction in the incorporation of Arm-F₂₆₀L GP into retroviral particles. We also observed that the F₂₆₀L mutant consistently suppressed the production of the retroviral vector particles, as measured by the amount of MLV CA protein in the supernatant (Fig. 3C). Taken together, these findings suggest that the single F₂₆₀L change

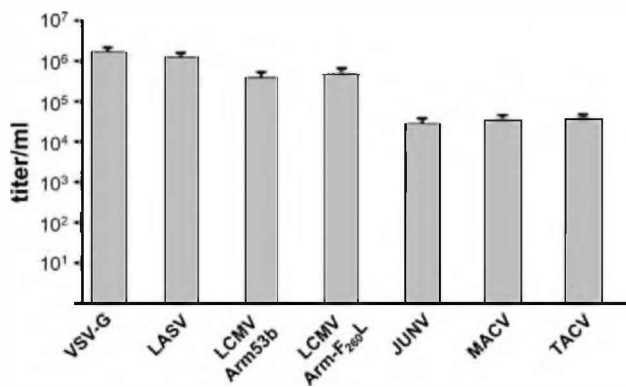


Fig. 2. Titers of pseudotyped retroviral vectors on 293A cells. Titer was determined by FACS analysis of cells to determine GFP expression, 48 h after transduction, and the values shown are the mean \pm SD for 3–6 independent experiments.

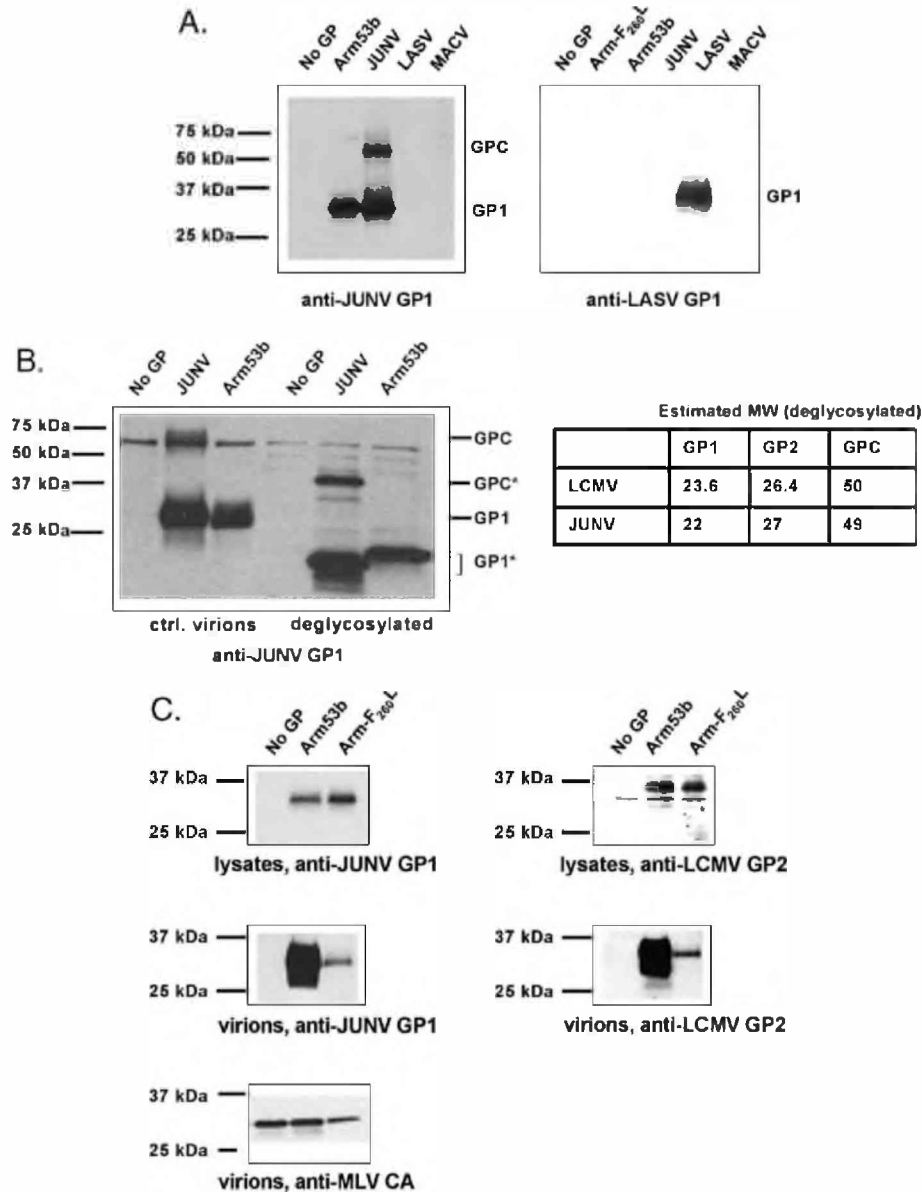


Fig. 3. Incorporation of arenavirus GPs into retroviral vectors. (A) Western blots of pseudotyped retroviral vector particles, probed with anti-JUNV GP1 antiserum or anti-LASV GP1 monoclonal antibody. The vector particles were concentrated by ultracentrifugation through a sucrose cushion. The positions of the GPC and GP1 subunits are indicated. (B) Western analysis of control and deglycosylated vector particles probed with the anti-JUNV GP1 antiserum. Comparison of the sizes of the deglycosylated proteins (GPC* and GP1*) indicates that the antibody recognizes the GP1 subunits. (C) Comparison of levels of Arm533b and Arm-F₂₆₀L GP in cell lysates and retroviral particles using anti-JUNV GP1 antiserum and anti-LCMV GP2 monoclonal antibody. Significantly less Arm-F₂₆₀L protein was incorporated into virions, while showing equivalent or slightly increased levels in the corresponding cell lysates. Expression of the Arm-F₂₆₀L GP also inhibited production of retroviral particles, as revealed by the anti-MLV CA antibody.

results in a protein that is more efficient at directing entry into cells, despite its adverse effects on incorporation into retroviral particles.

Differences in tropism of clade B and Old World pseudotyped vectors

We next expanded our studies to examine the ability of the vectors to transduce a variety of different cell types. To monitor for any cell-specific effects on titer that were directed against retroviral components, such as host restriction factors that

recognize the retroviral core (reviewed in Nisole et al., 2005), control vectors were included that were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G). We found that the titers obtained varied with both the nature of the GP and the cell type examined (Table 1). However, similar to our findings with 293A cells, in several cell types that were permissive for entry directed by all the GPs, we observed lower titers for the JUNV and MACV GP pseudotyped vectors than the Old World pseudotypes (HeLa, NIH3T3, mES, IEC-6 and CEF cells).

We noted that human T and B lymphocytes (CEM, Jurkat, Nalm-6 and Raji cells) were completely resistant to LASV and

Table 1
Titers^a of pseudotyped vectors on various cell lines

Glycoprotein	Human						Simian	Rodent			Avian	
	HeLa	K562	CEM	Jurkat	Nalm 6	Raji	Vero	NIH3T3	mES	CHOK1	IEC-6	CEF
VSV-G	8.0×10^4	2.5×10^4	8.5×10^5	6.5×10^5	2.0×10^5	2.4×10^4	4.7×10^5	4.8×10^4	2.2×10^5	2.4×10^5	2.1×10^5	3.9×10^5
Old World												
LASV	2.2×10^5	1.9×10^5	0	0	0	0	7.3×10^3	4.1×10^4	1.4×10^5	3.0×10^4	1.1×10^5	7.4×10^3
LCMV-Arm53b	2.8×10^5	6.3×10^3	0	0	0	0	1.4×10^3	4.4×10^4	4.7×10^4	6.1×10^3	1.1×10^4	4.5×10^3
LCMV-Arm53b(F _{260L})	2.0×10^5	4.4×10^3	0	0	0	0	2.2×10^3	5.6×10^4	5.7×10^4	8.3×10^3	1.8×10^3	8.0×10^3
New World clade B												
JUNV	8.0×10^3	1.3×10^4	1.8×10^4	3.2×10^3	1.4×10^2	0	2.1×10^3	4.3×10^3	5.9×10^3	1.2×10^3	4.0×10^3	9.8×10^2
MACV	3.0×10^4	1.3×10^3	3.1×10^4	2.2×10^3	2.5×10^3	2.7×10^2	8.7×10^3	1.6×10^2	2.2×10^2	2.1×10^2	1.6×10^2	8.2×10^2

^a Titers of pseudotyped retroviral vectors were measured by FACS analysis of transduced cells, 48 hrs post-transduction, and are shown as number of GFP-positive cells per ml of unconcentrated vector supernatant. Values shown are averages of 2–8 independent experiments.

LCMV vectors, while supporting reasonable titers for the JUNV and MACV vectors. It has previously been reported that both human and mouse lymphocytes are resistant to infection by LCMV (Borrow and Oldstone, 1992; Imperiali et al., 2005), probably because of incorrect glycosylation of α -DG in these cells (Imperiali et al., 2005). The mouse T lymphocyte cell line TIB-27 was also found to be resistant to transduction by LCMV GP pseudotyped vectors (data not shown). Our data both confirm and extend these findings by demonstrating that this restriction can be conferred solely by the GP.

Lack of transduction of lymphocytes by Old World vectors occurs despite the presence of dystroglycan

A possible explanation for the lack of titer of the Old World vectors on human lymphocytes is that the cells do not express sufficient quantities of the presumed Old World receptor, α -DG. We addressed this possibility by performing Western blot analysis to examine the levels of dystroglycan on cells that were both permissive and non-permissive for transduction. However, analysis of the levels of α -DG proved technically difficult and we were unable to find an antibody that specifically and unequivocally detected the α -DG subunit on the complete panel of cell lines we looked at, despite using three different antibodies, I1H6, VIA4-1 and 1B7 (data not shown). To examine the levels of dystroglycan, we therefore resorted to using an anti- β -DG antibody, 43DAG/8D5, raised against the extreme C-terminus of the human DG sequence. α -DG and β -DG are cleaved from the same precursor protein and remain associated by non-covalent interactions (Ibraghimov-Beskrovnya et al., 1992). For several of the cell lines tested, we observed 3 bands at

about 43, 37 and 25 kDa (Fig. 4). A similar pattern has also been observed in several carcinoma cell lines (Losasso et al., 2000; Muschler et al., 2002), where the lower molecular weight bands are thought to arise by proteolytic processing of β -DG (Losasso et al., 2000; Yamada et al., 2001). Using this antibody, we observed that all of the cell lines tested expressed β -DG and that there was no correlation between the ability of the arenavirus pseudotypes to enter cells and the overall levels of β -DG expression. A similar conclusion was reached in a recent study (Imperiali et al., 2005).

Despite the fact that we detected β -DG in human lymphoid cell lines, we noted that the lymphocytes had lower levels than the other human cell types (HeLa, 293A), suggesting the possibility that the levels of DG could be suboptimal for function as an arenavirus receptor in these cell types. Accordingly, we transduced CEM cells with a retroviral vector, pBabeDG, which contained the DG cDNA cloned into vector pBabeNeo (Morgenstern and Land, 1990). Following confirmation that this treatment increased the levels of DG in both CEM and control 293A cells (Fig. 5), we challenged the CEM-pBabeDG population with LASV and LCMV-Arm53b pseudotyped vectors. However, we still observed no transduction (data not shown).

We also examined whether CEM cells secreted an inhibitor of cell entry, such as soluble α -DG that could bind to the GP and interfere with subsequent cell binding and entry. However, incubation of 293A cells with supernatant from CEM cells had no effect on the titer of LASV or Arm53b pseudotyped vectors (data not shown).

Finally, we examined whether lymphocytes could be refractory to entry by Old World GP due to the presence of an inhibitory factor. We generated heterokaryons between non-

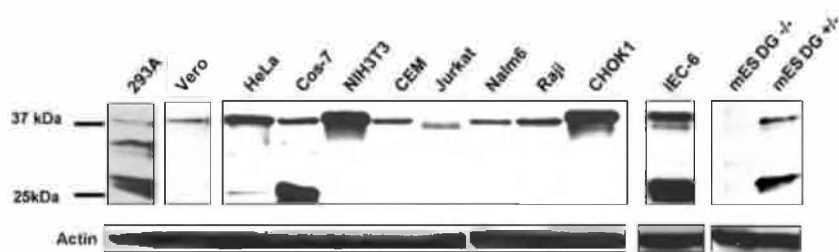


Fig. 4. β -DG expression in various cell types. Twenty micrograms of cell lysates was loaded in each lane, and the Western probed with anti- β -DG antibody. mES are murine ES cells, either knockout for DG expression ($-/-$) or the parental $+/-$ cell line.

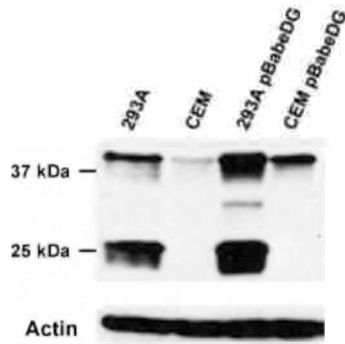


Fig. 5. Over-expression of DG. 293A or CEM cells were transduced with retroviral vector pBabeDG, and the levels of DG examined 48 h later using Western blotting and an anti- β -DG antibody. A significant increase in expression was observed in both cell types.

of α -DG, and specifically O-mannosylation, is essential for its activity as a receptor for LCMV (Imperiali et al., 2005; Kunz et al., 2005) is in good agreement with our findings.

Requirement for α -DG in mouse ES cells

Our data so far strongly suggest that the New World clade B viruses use a different receptor(s) than the Old World viruses. In order to examine the requirements for α -DG in more detail, we compared the titers of pseudotyped retroviral vectors on both DG knockout ($-/-$) and control heterozygous ($+/-$) murine ES cells (Williamson et al., 1997). Standard retroviral vectors are very inefficient at transducing murine ES cells, so it was necessary to perform these experiments using MND-based retroviral vectors. These vectors contain alterations in the long terminal repeat and 5' untranslated regions of the MLV genome that combine to increase transduction of murine ES cells (Challita et al., 1995).

We determined the titer of a panel of pseudotyped MND vectors on the DG $+/-$ and $-/-$ murine ES cells. Since retroviral vectors frequently give different titers when used to transduce individual clones derived from the same population, presumably due to a number of factors that can affect the efficiency of transduction, we included VSV-G pseudotyped vectors as a positive control. Although the cell surface receptor for VSV-G is not clearly defined, VSV-G is not expected to be affected by the presence or absence of α -DG. Using this control, we observed consistent differences in titer for the VSV-G pseudotyped vectors on the two cell lines, with the titers on

permissive CEM and permissive 293A cells by labeling each cell type, fusing the cells with PEG and FACS-sorting for the doubly labeled heterokaryons, as described (Abada et al., 2005). Subsequent challenge with LASV and Arm53b GP pseudotyped vectors demonstrated that the heterokaryons could be readily transduced by both vector types (Fig. 6). This finding rules out the presence of a dominant negative factor in the CEM cells that could act to inhibit the entry of the Old World viruses and instead suggests that these cells are simply lacking expression of a functional form of a necessary receptor or co-receptor molecule. The recent suggestion that the correct glycosylation

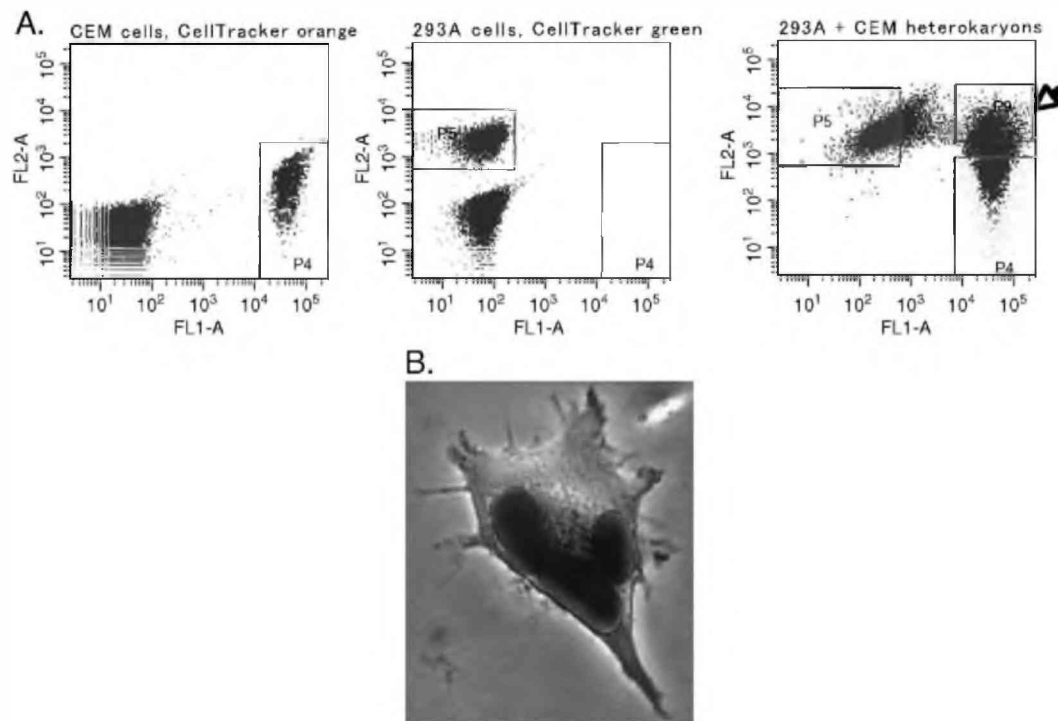


Fig. 6. Heterokaryons formed between CEM and 293A cells are transduced by LASV GP pseudotyped vectors. (A) CEM and 293A cells were stained with CellTracker orange and green, respectively, fused with PEG, and the doubly staining heterokaryons isolated by FACS (arrowed population). (B) A typical transduced cell from the heterokaryon population following X-gal staining. Several dark blue staining nuclei are present in each cell, resulting from the expression of nuclear localized β -galactosidase carried by the LASV GP vectors.

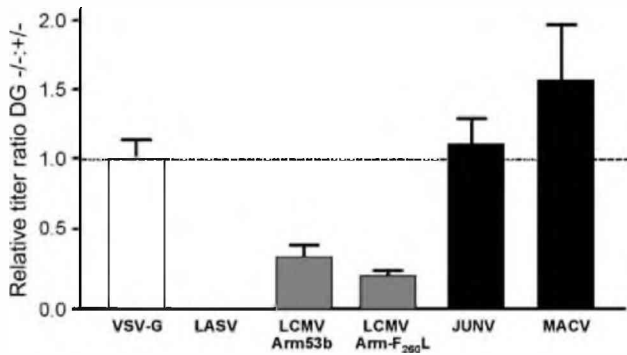


Fig. 7. Effect of DG expression in murine ES cells on GP-directed titer. Titers of GP pseudotyped vectors were measured on DG $-/-$ and $+/-$ murine ES cells and the ratios determined. To correct for the different susceptibility of the two cell lines to transduction by retroviral vectors, all ratios were made relative to the VSV-G control in each experiment. Data shown are the mean \pm SD for >5 independent experiments.

the $-/-$ cells being on average 55% of the level obtained on the $+/-$ cells. To compensate for this and to allow direct comparison of the effect of the GP on titer between the $-/-$ and $+/-$ cells, we made all titers relative to the VSV-G control on the same cell line (Fig. 7).

These analyses revealed several interesting findings. First, we observed striking differences among the Old World viruses in their requirements for α -DG. While LASV GP had an absolute requirement for DG in murine ES cells, the two LCMV GPs were only somewhat affected, suggesting that LCMV can efficiently use an alternate receptor(s) to gain entry into murine ES cells. Previously, it has been reported that the clone 13 strain of LCMV has a significantly greater requirement for α -DG than the parental Arm53b strain, and our data agree with these findings since the F₂₆₀L mutant was more sensitive to the loss of DG than Arm53b ($P = 0.027$). However, the fact that we still observed entry for vectors pseudotyped with the F₂₆₀L mutant GP suggests that there may be differences between the GP pseudotyped vector system and the native virus that we are not aware of or could simply reflect a difference in sensitivity in the two systems.

This analysis also revealed that the clade B JUNV and MACV GPs do not require α -DG for entry since the ratio of titers on the two cell types was similar to the VSV-G control. These data agree with our findings of broad tropism for the clade B GP vectors on the panel of cell lines tested, including their ability to infect lymphocytes. Together, they demonstrate that the clade B viruses use receptor(s) other than α -DG.

Discussion

The identification of the cell surface receptor used by an enveloped virus can help to explain its tropism and increase our understanding of its pathogenicity and disease characteristics. In the arenaviruses, α -DG was previously identified as a receptor used by LASV, certain strains of LCMV and the New World clade C viruses (Cao et al., 1998; Spiropoulou et al., 2002). Although it is likely that LCMV can use other receptor

molecules and that the clade A and B New World viruses do not use α -DG (Spiropoulou et al., 2002), no other molecules have been proposed as receptors for this family.

Previous studies of arenavirus receptors have been performed using a variety of in vitro assays, including binding assays and VOPBAs, together with virus infections using α -DG knockout murine ES cells (Borrow and Oldstone, 1992; Cao et al., 1998; Kunz et al., 2004; Smelt et al., 2001; Spiropoulou et al., 2002). The pseudotyped vector system that we have used in this work has distinct advantages for analyzing virus entry and receptor usage, including the ability to analyze the role of GP in the absence of other viral proteins. Importantly, these analyses can be performed under BSL-2 conditions, and similar systems have been widely used for other pathogenic enveloped viruses (Bartosch et al., 2003; Lin and Cannon, 2002; Wool-Lewis and Bates, 1998). Since the read-out in this system is an entry event (transduction), it is also likely to be a more accurate measure of functional GP–receptor interactions than can be obtained using in vitro assays since these are not always predictive of entry into cell lines. For example, the LCMV isolate PBL364 shows high affinity binding to α -DG in vitro yet has almost wild-type kinetics of infection on α -DG $-/-$ murine ES cells (Kunz et al., 2004), and VOPBA assays performed on rat intestinal epithelial cells were negative for Arm53b binding (Borrow and Oldstone, 1992), despite our finding that Arm53b GP pseudotyped vectors can readily transduce these cells.

The pseudotyped retroviral vector system also has advantages when compared to studies of entry made using infectious virus. Apart from the biosafety issues involved, viral infections are a complex system, where variations in tropism can be caused by proteins other than the GP. In addition, the arenaviruses have a short replication cycle coupled to a high mutation frequency of approximately 10^{-3} to 10^{-5} misincorporations per nucleotide site and round of copying (Domingo and Holland, 1997), leading to the generation of a large population of variants (Ruiz-Jarabo et al., 2003). Consequently, the biological properties of the variants may be very different from the original isolate used to initiate a culture or inoculate a host. Finally, such studies do not rule out the possibility that the lack of replication in certain cell lines is due to other, post-entry blocks to the arenavirus life-cycle.

Our study confirms and extends previous findings reported for arenavirus receptor usage by the Old World viruses. In agreement with Kunz et al. (2005), we found that LASV has an absolute requirement for α -DG in murine ES cells. However, in the context of pseudotyped retroviral vectors, we observed that the GP from the prototypic Arm53b isolate of LCMV was still able to transduce α -DG $-/-$ murine ES cells at approximately 30% the level of cells expressing α -DG. Previous studies have also reported lower levels of infection for LCMV isolates in the absence of α -DG (Kunz et al., 2003; Smelt et al., 2001). However, introducing the mutation F₂₆₀L into the Arm53b GP, which mimics the change present in the clone 13 virus isolate, did not completely abolish transduction of $-/-$ cells, although the ratio of transduction on the $-/-$ to $+/-$ cells was significantly lower than observed for the Arm53b isolate, indicating a greater dependence on α -DG for efficient entry.

Overall, our data provide strong evidence for the existence of an alternate receptor on murine ES cells that can be used by LCMV but not by LASV. It is not clear at present whether this molecule represents a distinct receptor molecule in its own right or whether it normally functions as a co-receptor to α -DG that can be used, albeit less efficiently, in the absence of α -DG. Such a situation is known to exist in the primate immunodeficiency viruses, where the viral Env binds sequentially to both a primary receptor, CD4, and a co-receptor molecule such as CCR5, but where CD4-independent entry is observed for certain isolates of both SIV and HIV-1 (Dumonceaux et al., 1998; Edinger et al., 1997). Clone 13 is reported to display both higher affinity for α -DG and a greater dependence on α -DG for entry than the Arm53b isolate (Kunz et al., 2004; Smelt et al., 2001). These two attributes are consistent with a model whereby the F₂₆₀L substitution could make the GP more dependent on α -DG binding to allow subsequent co-receptor binding, and thereby making α -DG-independent entry more difficult for this strain.

The marked discrepancy in the behavior of the LASV and LCMV pseudotyped vectors on α -DG $-/-$ murine ES cells was not observed on any other cell type we examined, including other rodent cell lines (NIH3T3, IEC-6, CHO-K1). Instead, these Old World pseudotypes generally behaved in the same way, with titers falling in the same order of magnitude. Probably this reflects the dominance of α -DG as a receptor for these viruses.

For the Old World virus pseudotypes, we also consistently observed that T and B lymphocytes were non-permissive for entry. Recent work has demonstrated the importance of correct O-mannosylation of α -DG in order for it to serve as an arenavirus receptor (Imperiali et al., 2005; Kunz et al., 2005), and it is probable that the glycosylation state of DG in these cells underlies the block to arenavirus entry. Furthermore, binding studies that we have performed using a LASV GP1 immunoadhesin and the T lymphocyte cell line CEM revealed that GP1 does not bind to these cells (Reignier et al., unpublished observations), which is consistent with this hypothesis. Interestingly, the lack of transduction of lymphocytes by the Old World vectors suggests that the alternate receptor present on murine ES cells that LCMV GP can use is not available on either human or murine lymphocytes.

For the New World clade B viruses that we studied, entry into murine ES cells directed by the JUNV and MACV GPs was unaffected by the loss of α -DG. These two GPs displayed a very broad tropism, transducing human, simian, rodent and avian cell lines and were able to enter human lymphocytes. Together, these data suggest that the receptor(s) that they use are likely to be distinct from any alternate receptors that could be utilized by LCMV in the absence of α -DG. The ability of the JUNV and MACV pseudotypes to transduce human lymphocytes is at odds with their *in vivo* tropism, where cells of the monocyte/macrophage lineage are infected but not lymphocytes (Geisbert and Jahrling, 2004). However, since viral tropism results from a complex set of interactions between a virus and a host cell, the ability of the JUNV and MACV GPs to direct entry into a cell does not necessarily predict a productive infection in that particular tissue in a patient.

Overall, our study reveals that receptor usage by the arenaviruses may be more complex than was previously appreciated. The search for the clade B receptor(s) and the alternate or co-receptors used by LCMV is an important goal for understanding more about the pathogenicity of these viruses.

Materials and methods

Cell lines

293A, 293T, Vero, HeLa, Cos-7, NIH 3T3, CHOK1, TIB-27 and IEC-6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA), with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 2 mM glutamine (Gibco BRL, Grand Island, NY). Raji, Jurkat, CEM and Nalm-6 cells were maintained in RPMI (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS and 2 mM glutamine. Dystroglycan-null knockout ($-/-$) and heterozygous control ($+/-$) R1 murine embryonic stem (ES) cells (Williamson et al., 1997) were generously provided by Dr. Kevin Campbell, University of Iowa. These cells were grown in DMEM supplemented with 20% FBS, 2 mM glutamine, 1 mM non-essential amino acids, 0.001% β -mercaptoethanol and 10^3 units/ml of LIF (ESGRO, Chemicon, Temecula, CA). All cells were maintained in 5% CO₂ except the TIB-27 cells which were maintained in 10% CO₂.

Arenavirus glycoproteins

The GP from the Armstrong 53b (Arm53b) strain of LCMV (Southern et al., 1987) was kindly provided by Gabor Veres (Cell Genesys), expressed from the CMV immediate early promoter in vector pCI (Promega, Madison, WI). When compared to the original database sequences of Arm53b GP (accession number NP_6948514), two differences were found, R₁₇₇A and A₃₁₃E. These changes are also present in two other database sequences for LCMV GP (accession numbers AAX49341.1 and P09991) and were also observed in two independent isolates of Arm53b that we cloned from a virus stock kindly provided by Dr. M. Suresh (University of Wisconsin, Madison). The clone 13 variant of LCMV-Arm53b GP contains the substitution F₂₆₀L (Salvato et al., 1988), and this mutation was introduced into the Arm53b GP to generate Arm53b-F₂₆₀L. The GP from LASV (Josiah strain, accession no. AY628203) was expressed in vector pCAGGS (Niwa et al., 1991). A sequence corresponding to the reported database sequence of JUNV GP (Parodi strain, accession no. U70799) was derived from genomic fragments of the JUNV S strand (Albarino et al., 1997) and cloned into the CMV immediate early promoter expression plasmid pSA90 (Soneoka et al., 1995). Tacaribe virus (TACV) GP was cloned by RT-PCR from total RNA isolated from infected Vero cells and cloned into the CMV immediate early promoter expression vector pCAGGS. It differs from the database sequence for strain TRVL 11598 (accession number P31840) at two positions, R₂₃₃H and E₄₄₆R. The GP from MACV (Carvalho strain, accession no.

AY129248) was synthesized as a codon optimized open-reading frame and cloned into the pCAGGS.

Retroviral vector production and titer determination

Retroviral vectors displaying arenavirus GPs were produced by transient transfection of plasmids pCgp (Han et al., 1998), pMND-eGFP (Zheng et al., 2004) or pCnBg (Han et al., 1998) and the appropriate GP expression plasmid into 293T cells, essentially as described (Christodoulopoulos and Cannon, 2001; Han et al., 1998). The pCgp plasmid expresses murine leukemia virus (MLV) Gag-Pol, and plasmids pMND-eGFP and pCnBg are retroviral vector genomes expressing enhanced green fluorescence protein (eGFP) or nuclear-localized β -galactosidase, respectively, as reporter genes. Retroviral vector supernatants were harvested 48 h post-transfection filtered through a 0.45 μ m filter (Millipore Corp., Bedford, MA), and aliquots were stored at -80°C . Titers were determined by incubation of serially diluted vector stocks with target cells for 48 h followed by calculation of percent positive cells, either by X-gal staining for β -galactosidase expression (Christodoulopoulos and Cannon, 2001) or by FACS analysis for eGFP expression.

Western blot analysis

For detection of β -dystroglycan (β -DG), cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS] containing 5 mg/ml sodium deoxycholate, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride [Sigma]) at 4°C for 10 min, centrifuged in an Eppendorf microfuge at $16,000 \times g$ for 10 min and the cleared supernatants were diluted 1:1 in $2 \times$ SDS gel loading buffer (Biorad, Hercules, CA) plus 5% 2-mercaptoethanol, boiled for 10 min and electrophoresed in 8–16% polyacrylamide gels (Biorad). The proteins were transferred to an Immobilon P polyvinylidene fluoride transfer membrane (Millipore Corp., Bedford, MA) and blocked overnight at 4°C with blocking buffer (5% dried milk in PBST [PBS {pH 7.4}, 0.1% Tween 20]). β -DG was detected using the 43DAG1/8D5 antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) at a 1:50 dilution in blocking buffer followed by horseradish-peroxidase-conjugated goat anti-mouse IgG (1:10,000) (Pierce, Rockford, IL). Specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences Corp., Piscataway, NJ).

Viral GPs in either cell lysates or retroviral particles were detected by Western blot analysis of cell lysates or concentrated virus supernatants, essentially as described (Christodoulopoulos and Cannon, 2001). The antibodies used were the anti-LASV GP monoclonal antibody L52-161-6 (Ruo et al., 1991) at a 1:10,000 dilution, the anti-JUNV GP monoclonal antibody QD04 AF03 (Sanchez et al., 1989; York et al., 2005) at a 1:2000 dilution, the anti-LCMV GP2 monoclonal antibodies WE-83.6 (Parekh and Buchmeier, 1986) at 1:100 dilution and 2704 (Parekh et al., 1989) at a

1:3000 dilution. Retroviral vector particles were detected using the goat anti-Rauscher MLV CA antiserum (Quality Biotech, Camden, NJ) at a 1:10,000 dilution. Where required, viral proteins were deglycosylated by incubation with peptide N-Glycosidase F (PNGase F; New England Biolabs, Ipswich, MA) for 90 min at 37°C , using 750 units of enzyme for 20 μ g of total protein.

Heterokaryon analysis

CEM and 293A cells were stained by incubation for 45 min at 37°C in growth media containing 1 mM CellTracker green (Molecular Probes, Eugene, OR) for CEM cells, or 10 mM CellTracker orange (Molecular Probes) for 293A cells, essentially as described (Abada et al., 2005). A mixture of 1×10^7 of each labeled cell type was fused by the addition of 1 ml of 50% polyethylene glycol (PEG) with a molecular mass of 3000 Da (Sigma) in 2% glucose, as described (Abada et al., 2005). Following overnight incubation, the doubly labeled population was sorted using a FACS DIVA (Becton Dickinson, San Jose, CA) with an excitation laser frequency of 488 nm and emission detected at 525 nm. The sorted cells were plated in 6-well plates, and 48 h later, the heterokaryons were challenged with LASV or LCMV-Arm53b pseudotyped retroviral vectors expressing nuclear localized β -galactosidase (vector pCnBg). Staining with X-gal (Han et al., 1998) and microscopic examination were used to determine whether the heterokaryons had been transduced.

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