Characterization of Chikungunya pseudotyped viruses: Identification of refractory cell lines and demonstration of cellular tropism differences mediated by mutations in E1 glycoprotein

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ABSTRACT

Chikungunya virus (CHIKV) is an alphavirus responsible for a number of large outbreaks. Here we describe the efficient incorporation of CHIKV envelope glycoproteins into lentiviral and rhabdoviral particles. Vectors pseudotyped with CHIKV envelope proteins efficiently transduced many cell types from different species. However, hematopoietic cell types were either partially or completely refractory. A mutation in E1 (A226V) has been linked with expansion of tropism for mosquito species, although differences in in vitro infection of mosquito cell lines have not been noted. However, pseudovirion infectivity assays detected subtle differences in infection of mosquito cells, suggesting an explanation for the changes in mosquito tropism. The presence of C-type lectins increased CHIKV pseudotyped vector infectivity, but not infection of refractory cells, suggesting that they act as attachment factors rather than primary receptors. CHIKV pseudotypes will serve as an important tool for the study of neutralizing antibodies and the analysis of envelope glycoprotein functions.

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Introduction

Chikungunya virus (CHIKV) is an alphavirus belonging to the *Togaviridae* family and was first isolated in 1952 from a febrile individual in Tanzania (Peters, 1990; Strauss, 1986). CHIKV is transmitted to humans by several species of mosquitoes, with *Aedes aegypti* and *A. albopictus* being the two main vectors. Acute infection is characterized by a painful polyarthralgia, high fever, asthenia, headache, vomiting, rash and myalgia (Bodenmann and Genton, 2006; Peters, 1990; Pialoux et al., 2007). In many patients, a chronic and incapacitating arthralgia persists for months. During the last 50 years, there have been numerous CHIKV outbreaks in East and Southern Africa and in Southeast Asia (Schuffenecker et al., 2006). Though generally not fatal, CHIKV has sickened 1.6 million people in the Indian Ocean region since 2005 (Charrel et al., 2007). Three distinct phylogroups have been described based on E1 sequence of different CHIKV isolates, and microevolution of virus strains has been associated with infection outbreaks (Schuffenecker et al., 2006). In particular, changes in the envelope glycoproteins (gps) of the virus have been described that affect infectivity in different mosquito species. A single change at position 226 of E1 protein has been associated with the adaptation of the virus to more efficiently infect and be transmitted by *A. albopictus*, which is thought to be the major vector of the large scale CHIKV epidemic on La Reunion Island (Tsetsarkin et al., 2007; Vazeille et al., 2007), as well as a recent outbreak in Italy (Bonilauri et al., 2008). This change has also been shown to modulate cholesterol requirement for infection of insect cells by CHIKV (Tsetsarkin et al., 2007), as well as other alphaviruses (Ahn et al., 1999; Lu et al., 1999). Whether there is a correlation between cholesterol requirements and CHIKV fitness increase in *A. albopictus* remains unknown. The adaptation of the virus to the broadly distributed mosquito species *A. albopictus* increases the potential of CHIKV to extend its range further into Europe and America (Tsetsarkin et al., 2007).

Alphaviruses are small enveloped, single stranded, positive polarity RNA viruses (Peters, 1990). They attach to poorly characterized receptors on many different cell types in various species. Viral entry generally occurs through receptor-mediated endocytosis, and fusion is dependent on endosomal acidification (DeTulleo and Kirchhausen, 1998; Kolokoltsov et al., 2006; Marsh and Helenius, 2006; Marsh et al., 1984). CHIKV particles contain three structural proteins: glycosylated E1 and E2 envelope proteins, embedded in the viral membrane, and a non-glycosylated nucleocapsid protein. Based on similarity to other alphaviruses, E2 mediates receptor attachment, while E1 is a class II viral fusion protein. A third glycoprotein, E3, is not associated with mature virions but released into culture fluids (Simizu

et al., 1984), while 6K protein, a membrane-associated peptide created by cleavage of the polyprotein to release E2 and E1, is incorporated into particles at a low level (Gaedigk-Nitschko and Schlesinger, 1990; Lusa et al., 1991). There is a critical lack of knowledge of CHIKV biology, contrasting with related model alphaviruses like Sindbis virus (SINV), Semliki Forest virus (SFV), and Ross River virus (RRV). In particular, little is known about the interaction of CHIKV (and of most alphaviruses) with human primary cells. CHIK virus isolates from La Reunion outbreak have been described to infect *in vitro* primary fibroblasts, and, to a lower extent monocyte-derived macrophages, but not primary periferal blood mononuclear cells, primary lymphocytes and monocytes, nor monocyte-derived dendritic cells (Sourisseau et al., 2007). Studies using live CHIKV have been limited partially because they must be performed at biosafety level 3 (Anon, 2007).

Pseudotyped viruses have important applications as tools for the study of viral glycoprotein function and attachment/entry processes. They can also be used to analyze immune responses and the development of neutralizing antibodies. Furthermore, they can have utility as vaccines, as well as vectors for gene transfer and gene therapy. In particular, due to CHIKV's apparent preference for muscle satellite cells (Ozden et al., 2007), CHIKV may be useful for therapeutic gene replacement of muscle disease such as muscular dystrophy. Pseudotyped viruses incorporate heterologous viral gps thus acquiring the host range of the virus from which these gps are derived. Early steps in infection, such as receptor binding, membrane fusion, and entry, are determined solely by properties of the nonrelated envelope protein. Also virus envelope gps are the primary targets of neutralizing antibody responses. The epitopes recognized differ sufficiently between virus subtypes and species to distinguish viruses and provide an important basis for disease diagnosis.

Here we describe the successful development and validation of CHIKV pseudovirions. We have assessed CHIKV protein incorporation as well as sensitivity to sera from CHIKV infected individuals. Recapitulation of the cell tropism of the pseudovirions has been performed allowing us to describe several refractory cell lines. We also demonstrate that mutations in E1 glycoprotein mediate cellular tropism differences in mosquito cells. These pseudovirions permit analysis of the host range and function of the Chikungunya virus gps allowing future work that will help to elucidate the pathogenesis and tropism of this as yet poorly characterized virus. They will also improve laboratory safety and security as they can be handled at biosafety level 2.

Results

Chikungunya envelope glycoproteins are incorporated into lentiviral pseudoparticles

In order to determine if CHIKV envelope gps were incorporated into HIV pseudoparticles, 293T cells were transfected with pNL-luc, a lentiviral vector encoding luciferase, and a plasmid encoding the CHIKV envelope proteins with and without a point mutation in E1 (A226V) involved in mosquito tropism changes (Tsetsarkin et al., 2007). As controls, 293T cells were also transfected with plasmids encoding vesicular stomatitis virus (VSV) G protein and the pNL-luc vector. After 40 h, the virions obtained were concentrated by ultracentrifugation and the transfected cells were lysed. Both the concentrated virions and the cell lysates were analyzed by Western blot using a mix of 3 different sera from CHIKV infected individuals. CHIKV proteins E1 (52 kDa) and E2 (56 kDa) (that nearly co-migrate in the gel) as well as the p62 (62 kDa) precursor protein were detected in both wild type and A226V mutant cell lysates but not in the control VSV-G cell lysate (Fig. 1). Two nonspecific bands of approximately 80 and 50 kDa respectively were also observed in all the cell lysates samples (Fig. 1). Similar amounts of CHIKV envelope gps were also
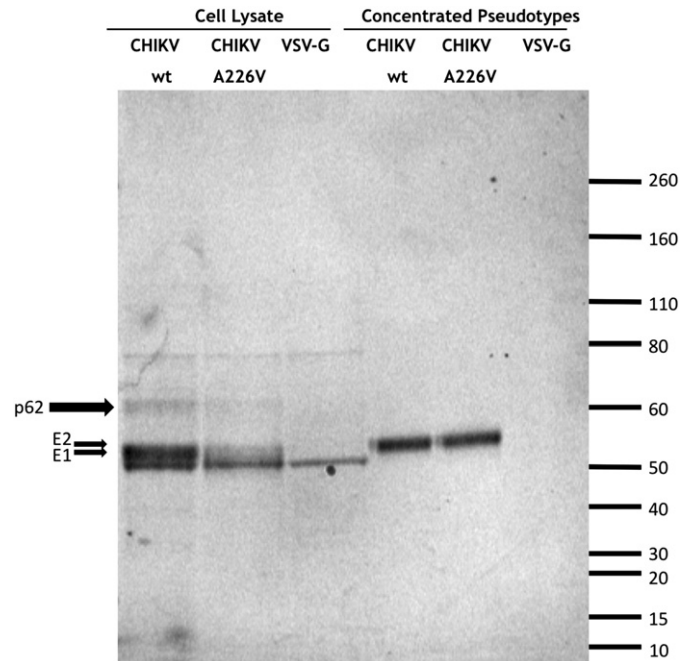

Fig. 1. Chikungunya virus envelope protein expression. Western blot was performed using a mix of human sera from CHIKV infected individuals to detect protein expression in pseudotype producer 293T cell lysates and concentrated HIV-pseudotypes bearing CHIKV wt and E1A226V mutant envelope glycoproteins and VSV-G protein. The positions of molecular mass markers (kDa) are shown on the right.

detected in the concentrated wild type and mutant CHIKV pseudoparticles indicating that lentiviral particle production was roughly equivalent in both transfections (Fig. 1). Thus, CHIKV gps are efficiently produced in 293T cells and are successfully incorporated into the HIV pseudoparticles. Also, the result demonstrates that the CHIKV proteins incorporated into the pseudoparticles are efficiently recognized by antibodies present in the sera of individuals with a prior history of CHIKV infection. Additionally, immunofluorescence studies using anti-CHIKV positive sera on live cells suggested the CHIKV gps were efficiently expressed on the surface of transfected cells (data not shown). Incorporation into murine leukemia virus (MLV)-based retroviral particles was also demonstrated with titers in RD muscle cell line of 9.8×10^6 FFU/ml for CHIKV envelope and 1.3×10^7 FFU/ml for VSV-G.

CHIKV pseudovirus infection is inhibited by sera from infected individuals

As expected from previous studies with replication competent CHIK (Ozden et al., 2007), pseudovirions carrying CHIKV envelope efficiently infected the muscle derived RD cells (1.4×10^7 FFU/ml). To further characterize the specificity of CHIKV pseudotype infection, we tested the ability of sera from individuals previously infected with CHIKV to neutralize wild type and A226V E1 mutant pseudovirion infectivity in RD cells. Similar titers of wild type and mutant CHIKV pseudovirions (15 pg of p24) were incubated with either serum from 9 different infected individuals or a control serum from an uninfected individual at various dilutions and then used to infect RD cells. All sera from CHIKV infected patients were able to inhibit RD cell infection by either wild type or mutant envelope pseudotypes with similar efficiency (Table 1 and Supplementary Fig. 1). Inhibition was dose dependent whereas a negative control serum had little effect, although partial neutralization was seen at the highest concentration (Table 1 and Supplementary Fig. 1). Similar data was obtained with four additional, geographically diverse, negative control sera (data not

Table 1
Neutralization of CHIKV pseudovirions.

Sera	CHIK WT[a]		CHIK A226V[a]	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
1	3030	455	4348	526
2	3125	417	3846	500
3	3125	455	3704	500
3317	6250	833	7692	1000
4102	4545	588	6250	714
4743	6667	833	8333	1000
92869	909	71	1000	71
92958	2083	133	1818	137
93137	2128	161	2564	133
Control[b]	56	<30	38	<30

[a] The values represent the serum dilutions interpolated from the dose–response curves as inhibiting 50 or 90% of luciferase read-out relative to control assays carried out in the absence of serum. < indicates no neutralization at the lowest dilution of serum tested (1 in 30).

[b] Representative uninfected negative control serum.

shown). No neutralization was seen with CHIKV patient serum using control pseudotypes carrying VSV-G (data not shown). These results demonstrate that entry of the pseudotyped viruses is mediated by the CHIKV gps and validates their utility to evaluate CHIKV antibody neutralization capacity.

CHIKV pseudovirus infection is inhibited by lysosomotropic agents

The effects of lysosomotropic agents in pseudovirion infection were studied to further analyze the CHIKV envelope-mediated entry process. These agents are known to raise the pH of the endosomal compartment, efficiently blocking infection by viruses dependent on a low-pH step for entry (Marsh and Helenius, 1989; Miller and Lenard, 1980; Ohkuma and Poole, 1978). We chose RD cells as targets for infection because they are susceptible to infection by both VSV-G lentiviral pseudotypes that exhibit pH-dependent entry and ampho-tropic MLV (MLV-A) envelope lentiviral pseudotypes, whose entry is pH-independent (McClure et al., 1990). Bafilomicin A, an inhibitor of vacuolar H+-ATPases responsible for acidifying endosomes, inhibited transduction of RD cells by both CHIKV and VSV-G pseudotypes (Fig. 2). In contrast, no inhibition was detected in MLV-A pseudotype challenged cells, even with high bafilomicin A concentrations (Fig. 2) indicating that the inhibitory effects of bafilomicin A did not result from post-entry inhibition of HIV core replication. Similar results were obtained with the acidotropic weak bases ammonium chloride (NH_4Cl) and chloroquine (data not shown). Together, these data indicate that CHIKV envelope-mediated entry is dependent on endosomal acidification.
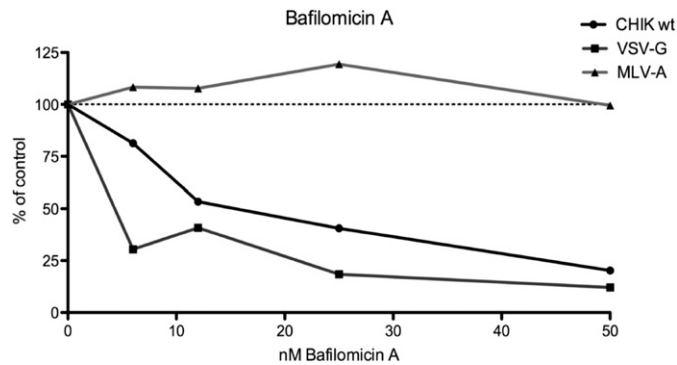

Fig. 2. Effect of lysosomotropic agents on pseudotype transduction. Bafilomicin A inhibition of RD cell transduction by MLV-A env (triangles), CHIKV gps (squares) and VSV-G (dots) pseudotypes. Results are expressed as percentage of no drug and represent the means of duplicate wells. The data is representative of four experiments.

Analysis of CHIKV pseudovirus tropism in mammalian cells

A panel of human adherent and non-adherent cell lines, primary human cells and non-human cell lines was used to characterize the tropism of CHIKV pseudotyped viruses (Table 2). Parallel infections were carried out with HIV particles produced in the absence of a viral envelope protein as a negative control and VSV-G pseudotyped particles, which display a very broad tropism, to distinguish from possible post-entry blockage effects. In this case the HIV vector used was pNL-gfp that carries the *Aequorea victoria* green fluorescent protein (GFP) gene as a reporter. Virus input of ultracentrifuge pelleted samples was normalized by p24 content prior to infection. The viral titers obtained on the individual cell lines varied quite dramatically (Table 2), but in general this variation was consistent between CHIKV envelope and VSV-G pseudovirions (Fig. 3). GFP expression showed that most cell types listed in Table 2 were permissive for CHIKV pseudotypes demonstrating the wide cellular tropism conferred by CHIKV envelope gps (Fig. 3). However most hematopoietic cell lines were either completely or partially refractory to CHIKV pseudotype infection. Primary, PHA/IL-2 stimulated PBLs were also not infectible by CHIKV pseudovirions. For reasons of better detection, vectors encoding murine CD24 were used in the place of GFP. PBLs were over 99% CD3 positive, and could be easily transduced with pseudovirions bearing VSV-G, resulting in 10.3% of cells staining positive for murine CD24. In contrast, even using 10-fold higher inoculum, CHIKV failed to give any significant transduction above that for no envelope controls (0.25 and 0.15% respectively).

Table 2
HIV pseudovirus titers determined by GFP expression.

Name	Cell type[a]	Virus titer (FFU/ml)[b]	
		CHIKV ENV	VSV-G
RD	Muscle	1.4×10^7	2.5×10^7
HT1080	Fibrosarcoma	1.4×10^7	1.9×10^7
SW480	Colorectal	1.2×10^7	2.4×10^7
293T	Kidney epithelial	7.7×10^6	2.6×10^7
NP2	Brain	3.1×10^7	2.4×10^7
HeLa	Cervical carcinoma epithelial	5.0×10^6	6.7×10^6
Hos	Bone	2.6×10^7	4.1×10^7
A549	Alveolar epithelial	1.7×10^7	3.0×10^7
Keratinocytes	Primary keratinocytes	7.4×10^7	8.3×10^7
Endothelial	Primary endothelial cells	2.6×10^7	3.4×10^7
Muscle	Primary muscle cells	3.4×10^7	ND

Name	Cell type[a]	Virus titer (FIU/ml)[c]	
		CHIKV ENV	VSV-G
THP1	Monocytes	4.0×10^4	3.1×10^5
BTHP1	B cell line	–[d]	1.1×10^5
HEL	Erytroleukemia	3.7×10^4	2.5×10^5
H9	CD4 T cell line	–[d]	1.3×10^5
MEG-01	Megakaryoblast	2.1×10^4	6.1×10^5
U937	Promonocytic	1.6×10^4	1.5×10^5
221-CD4	Monkey T cell line	–[d]	1.7×10^5
Macrophages	Primary monocyte-derived macrophages	3.9×10^4	6.4×10^4
MDBK	Bovine kidney	4.5×10^4	8.7×10^4
3T3	Mouse fibroblast	8.9×10^4	1.2×10^5
vEPT	Rabbit kidney	5.0×10^4	1.9×10^5
SK-RST	Pig kidney	1.2×10^5	5.2×10^5
Vero	Monkey kidney	9.0×10^4	2.0×10^5
BHK21	Hamster kidney	1.3×10^5	3.7×10^5
NRK	Rat kidney	1.7×10^5	5.1×10^5
CEF	Chicken embryo fibroblasts	1.2×10^5	1.3×10^5
QT6	Quail fibroblast	8.1×10^4	7.5×10^5
C6	Rat brain	3.0×10^5	7.3×10^5
L2	Rat lung	9.1×10^4	2.0×10^5

Results are representative of at least 2 independent experiments.

[a] Cells are human unless otherwise stated.

[b] Titer obtained by counting GFP positive cells under fluorescence microscope.

[c] Titer obtained by sample FACS analysis.

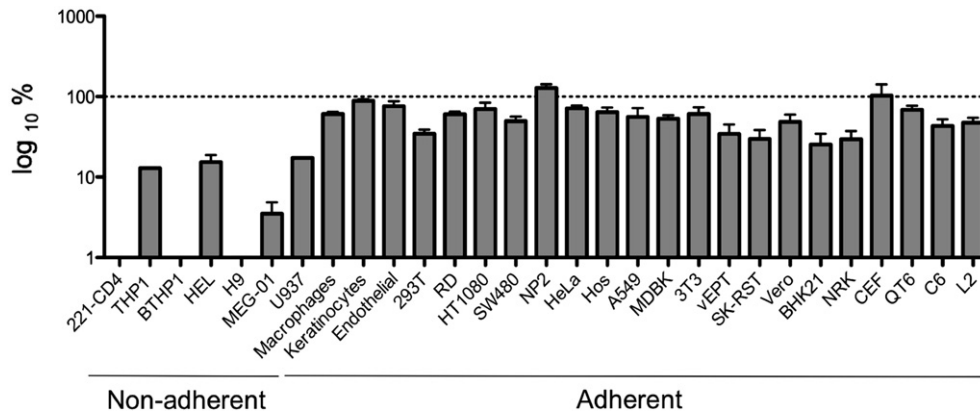[d] FACS data below negative ($<1 \times 10^4$ FIU/ml).

Fig. 3. Relative cell tropism of CHIKV pseudotypes. GFP expression was used for parallel titration of CHIKV and VSV-G pseudotypes in a panel of cell types. Relative CHIKV pseudotype titer as compared to VSV-G pseudotype titer in each cell type is shown. The dotted line indicates 100% infectivity for VSV-G pseudotypes. Data represent the average of at least two independent experiments.

The fact that hematopoietic cells were not infectible, suggested that there might be a correlation between cell adhesion and susceptibility. However, no difference in infectivity was found between adherent and suspension cultured 293FR cells (data not shown) indicating that the susceptibility to CHIKV pseudotype infection is most likely independent of cell adhesion. The titers of the VSV-G pseudotypes were consistently higher than that of the CHIKV envelope except in the case of human NP2 glioma cell line and chicken embryo fibroblasts in which CHIKV envelope pseudotypes titers were respectively 26% and 35% higher than those of VSV-G pseudotypes (Fig. 3). This is consistent with a strong tropism for fibroblasts and the CNS observed in in vivo models of CHIKV infection (Couderc et al., 2008). Endothelial human primary cells and human primary keratinocytes were also susceptible to CHIKV pseudotype infection (Fig. 3). In a separate experiment, fetal primary CD146+ myoblasts were also highly susceptible to CHIKV pseudotypes, with a titer of 4.3×10^7 FFU/ml. CD146 is a marker for myoblasts (Cerletti et al., 2006), and thus these results are consistent with previous findings that CHIKV infects muscle precursor cells (Ozden et al., 2007). It has been described that monocytes are insensitive to CHIKV infection while human primary macrophages are productively infected (Sourisseau et al., 2007). Differentiation of THP1 monocytes into macrophages using retinoic acid (Hickstein et al., 1992) resulted in a 4-fold increase in CHIKV pseudotype susceptibility (data not shown) suggesting that CHIKV inability to infect monocytes may be at least partially related to a lack of efficient entry that is overcome by the changes induced after the macrophage differentiation process. HIV does not replicate efficiently in primary monocytes, thus we developed VSV-based pseudovirions (Matsuura et al., 2001) in order to study CHIKV envelope-mediated monocyte infection. Experiments were performed with fresh isolated PBMC, containing primary lymphocytes and monocytes. While good infection was observed in CD14 positive cells (monocytes) using VSV-G envelope as a control with 8.8% of cells were GFP positive, little significant infection (1% of cells were GFP positive) was seen with the same backbone and CHIKV envelope.

Naturally occurring CHIKV variants carrying an alanine to valine change in E1 (A226V) have been described to alter vector specificity by increasing infectivity for the *A. albopictus* mosquito (Tsetsarkin et al., 2007; Vazeille et al., 2007). Whether this change has any effect in the ability of the virus to infect the mammalian host has not previously been addressed. We therefore compared the infectivity of both wild type and E1 A226V mutant pseudoparticles in different mammalian cell lines. Input was normalized by p24 ELISA analysis prior to infection so that the different cell lines were challenged with equal amounts each pseudotype. Statistical analysis showed no significant difference ($P>0.3$) in infectivity between CHIKV envelope variants in any of the cell lines tested (Fig. 4). This result indicates that the presence or absence of the change A226V in CHIKV E1 gp does not affect the gross ability of pseudotypes to infect mammalian cells, although we cannot rule out the possibility of changes in the kinetics of infection.

Mutations in E1 glycoprotein mediate cellular tropism differences in mosquito cells

As lentiviral pseudotypes were not sufficiently infectious in mosquito cells we decided to pseudotype VSV with CHIKV envelope to determine the effect of the A226V mutation in mosquito cell entry. Both *A. albopictus* cell lines and *A. aegypti* primary cells were tested for susceptibility to CHIKV pseudotyped VSV. Again, parallel infections were carried out with VSV particles produced in the absence of a viral envelope protein as a negative control and VSV-G pseudotyped particles to distinguish from possible post-entry blockage effects. Using particles first normalized by titering on RD cells, the infectivity of VSV pseudotypes bearing the CHIKV envelope 226V mutation was higher by more than 4 fold in C6/36 and 2 fold in C7–10 *A. albopictus* cell lines when compared to wild type envelope pseudotypes (Fig. 5A). These subtle differences were statistically significant ($P<0.05$) in multiple independent experiments using different batches of pseudovirions, and demonstrate a role for this E1 mutation in virus
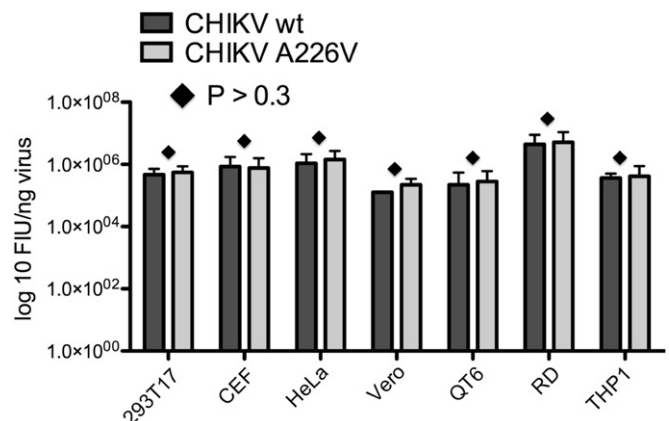

Fig. 4. Pseudotype infectivity for wt and A226V CHIKV envelopes in mammalian cells. Equal amounts (normalized by p24 ELISA) of pseudotypes bearing CHIKV envelope wt or 226V mutant were titrated in parallel in different cell types. GFP titer was determined by flow cytometry. Each pseudotype was tested in triplicate for each cell type and error bars represent standard deviations (SDs). Differences in viral titers were analyzed by pairwise *t*-tests. Diamond indicates $P>0.3$.
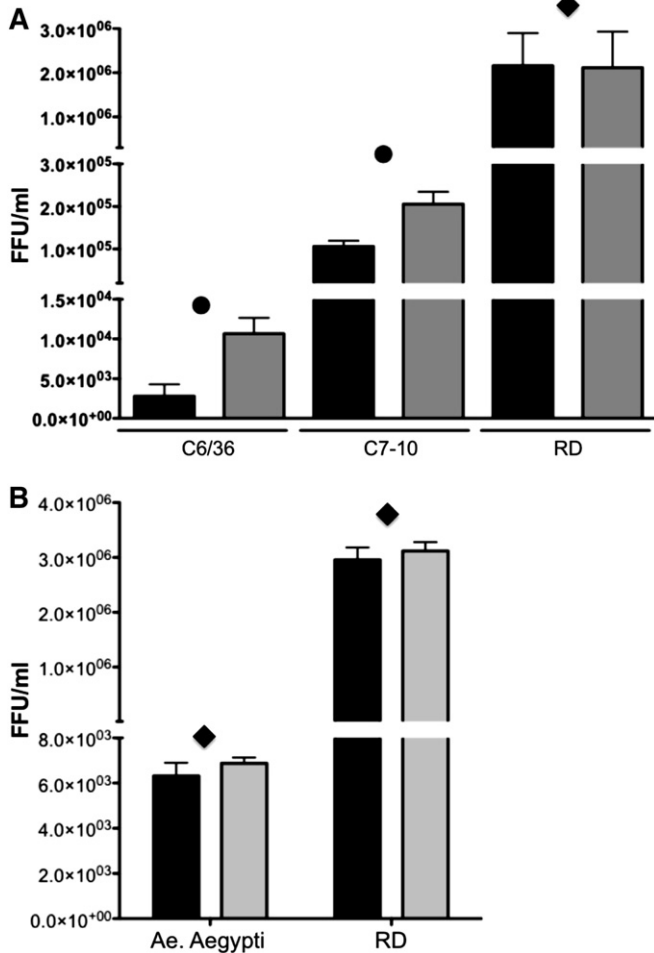
Fig. 5. Pseudotype infectivity for CHIKV envelope variants in mosquito cells. VSV pseudotypes bearing CHIKV envelope wt (Black bars) or 226V mutant (Grey bars) were titrated in parallel in (A) *A. albopictus* cell lines C6/36 and C7–10 and RD cells. Four replicates were performed for each pseudotype. (B) *A. aegypti* cells and RD cells. Each pseudotype was tested in triplicate. GFP titer was determined by flow cytometry. Error bars represent standard deviations (SDs). Differences in viral titers were analyzed by pairwise *t*-tests. Diamond indicates $P<0.3$. Dot indicates $P<0.05$.

binding/entry giving CHIKV 226V a selective advantage in *A. albopictus* cells. However, no such advantage is detected in *A. aegypti* cells since no differences in infectivity were observed between CHIKV wild type and 226V mutant in three independent experiments (Fig. 5B). The differences in CHIKV infectivity between C6/36 and C7–10 cell lines observed in Fig. 5A are not specific since similar differences were observed with the control VSV-G enveloped pseudotypes (8.6×10^3 FFU/ml in C6/36 and 1.3×10^5 FFU/ml in C7–10). No pseudotype induced cytopathic effects were observed after 24 h when the analyses were performed.

CHIKV pseudovirion infectivity is enhanced by the presence of C-type lectins

C-type lectins like DC-SIGN and DC-SIGNR have been shown to augment infection of many viruses by interacting with the envelope viral gps (Lozach et al., 2007), playing an important role in virus transmission, tissue tropism, and pathogenesis. To determine the role of lectin expression on the infectivity of CHIKV pseudotypes, we utilized a previously reported 293T-REx cell line system, in which lectin expression was under the control of a tet repressor protein that allows induction of expression with doxycycline (Pohlmann et al., 2001). The induction of lectin expression was verified by fluorescence-activated cell sorter staining (Supplementary Fig. 2) and Western blot

(data not shown). We challenged the lectin expressing 293T-REx cells with luciferase reporter pseudotypes bearing CHIKV envelope glycoproteins. Ebola Zaire (EboZ-GP) envelope and VSV-G pseudotypes, whose infectivity is respectively enhanced and unaffected by the presence of lectins (Simmons et al., 2003) were used as positive and negative controls. Infectivity of EboZ-GP envelope pseudotypes was enhanced over 35-fold in DC-SIGN-expressing 293T-REx cells and 26-fold in DC-SIGNR expressing cells, compared with the parental 293T-REx cells (Fig. 6). Similarly, the infectivity of CHIKV envelope pseudotypes was enhanced over 10-fold in DC-SIGN-expressed 293T-REx cells and 19-fold in DC-SIGNR expressed cells (Fig. 6). In contrast, VSV-G infection of either DC-SIGN or DC-SIGNR expressing cells was within 2-fold of parental 293T-REx cell infection. Enhancement of CHIKV pseudotypes infectivity was also observed in susceptible HeLa and RD cell lines expressing DC-SIGN and DC-SIGNR (data not shown). While expression of DC-SIGN on already susceptible cell lines enhanced infection, refractory lines, such as B-THP-1, NC-37 and Ramos cell lines, remained resistant to infection, despite stable DC-SIGN expression (data not shown).

Discussion

CHIKV characteristics and the widespread distribution of its mosquito vectors make it a potentially highly important emerging pathogen. However, very little is known about the biology of this alphavirus, although its similarity to other alphaviruses suggests general principles that may govern its biological activities. The envelope proteins are the major determinants of alphavirus cell tropism, viral entry and neutralization sensitivity. With the advancement of molecular technologies, several pseudotype reporter viral systems have been developed for different enveloped viruses making the investigation of phenotypic properties of envelope proteins more convenient and efficient (Bartosch et al., 2003; Chan et al., 2006; Fukushi et al., 2005; Ma et al., 1999; Matsuura et al., 2001; Rollman et al., 2007; Simmons et al., 2004; Wool-Lewis and Bates, 1998).

We have successfully exploited the cell surface expression of CHIKV proteins in transfected cells to produce lentiviral particles that are able to transduce cells in a CHIKV envelope-mediated manner. The infectivity of these pseudotyped reporter viruses in CD4 and CXCR4 negative cell lines (Table 2) with a single round of infection, indicates that the CHIKV envelope is directing entry, thus broadening the cell tropism of the resulting HIV particles. CHIKV gps are not only efficiently produced and expressed on the cell surface but are also incorporated in the pseudotyped viruses. Although E1 and E2 proteins
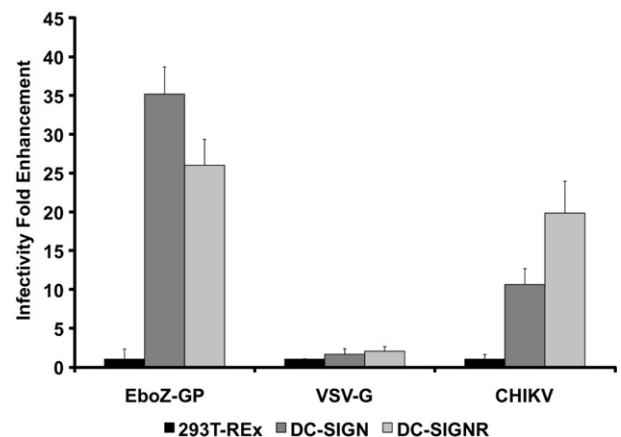

Fig. 6. DC-SIGN and DC-SIGNR enhance infectivity of CHIKV envelope glycoprotein-bearing pseudotypes in 293T-REx cell lines. The data are presented as fold-enhancement of infection in lectin expressing 293T-REx cells compared to parental 293T-REx cells. Error bars indicated standard deviations (SDs) of triplicate values from a representative experiment. Similar results were obtained in three independent experiments.

migrate closely on gels (Sourisseau et al., 2007) and could not be distinguished by size due to heterogeneous glycosylation, the fact that correct E2 processing requires E1 coexpression makes it likely that both proteins are being expressed. Furthermore, CHIKV pseudovirions present native epitopes on their surfaces that are recognized by neutralizing human sera from individuals previously infected with CHIKV. CHIKV pseudotypes enter cells through a pH-dependent pathway, being readily blocked by three inhibitors of endocytic acidification as described for CHIKV (Sourisseau et al., 2007). Thus, CHIKV lentiviral pseudotypes offer the potential to rapidly and easily study native entry pathways of the parental CHIKV in the absence of other cytotoxic effects associated with alphavirus infection.

The host range conferred by CHIKV envelope is very broad, enabling the infection by CHIKV pseudotypes of a variety of cell lines from diverse species and tissues. The infection of many of the cell types used for this work is not unexpected as CHIKV displays a rather wide tropism and has been shown to infect a variety of human and non-human cell types (Glasgow, 1966; Hahon and Zimmerman, 1970; Ozden et al., 2007; Rinaldo et al., 1975; Simizu et al., 1984; Sourisseau et al., 2007). Interestingly, human cell lines A549 and U937, recently described as resistant to CHIKV infection (Sourisseau et al., 2007) are susceptible to infection by CHIKV lentiviral pseudotypes. These results, together with the fact A549 cells are able to bind CHIKV virions (Sourisseau et al., 2007), indicate that the restriction to CHIKV replication in these cells occurs at a post-entry step of viral infection. The exception for CHIKV pseudotypes susceptibility was hematopoietic cell lines, most of which were partially or completely refractory to the infection (Table 2 and Fig. 3). Similar results have been described for replication competent CHIKV, as well as pseudovirions bearing envelopes from the related alphaviruses RRV and SFV (La Linn et al., 2005; Strang et al., 2005). We believe that the failure to transduce those cells is most likely due to the lack or limited expression of the cellular receptor(s), or cellular events involved in internalization, given the efficiency of transduction by VSV-G pseudotypes. These results suggest that CHIKV receptor(s) are present in different host species and thus CHIKV envelope provides a broad specificity to the pseudotyped vectors. Susceptibility of some cell types to Ebola virus has been described to be affected by cell adhesion properties (Dube et al., 2008). Our results show that the differences in CHIKV pseudotypes infectivity between adherent and non-adherent cells are independent of the anchorage-dependency of the cells. The refractory hematopoietic cell types may be useful for receptor identification. The fact that partially refractory THP1 monocytes increase their susceptibility upon differentiation to macrophages, most likely by induced expression of the virus receptor, makes cells of the monocyte/macrophage lineage potential candidates in aiding the discovery of the CHIKV cell surface receptor(s).

The use of CHIKV pseudotypes also provides a straightforward tool for the functional characterization of envelope gps variants. Single amino-acid changes in CHIKV gps such as E1 A226V have been described to affect virus specificity (Tsetsarkin et al., 2007; Vazeille et al., 2007). Moreover, adaptation of CHIKV 226V to *A. albopictus* mosquitoes coincides with CHIKV dependence on cholesterol in the target cell membrane (Tsetsarkin et al., 2007). Similarly, a mutation in the same position of the E1 gp from SFV has been described to affect the cholesterol dependency of the virus (Vashishtha et al., 1998) and, unlike CHIKV (Tsetsarkin et al., 2007), results in the more efficient growth of SFV in *A. albopictus* mosquitoes (Ahn et al., 1999). Whether there is direct correlation between the release from the cholesterol dependence, and the growth advantage in *A. Albopictus* remains unknown. As expected, we have found no effect of E1 A226V mutation in CHIKV pseudotypes infectivity in mammalian cells. However, the presence of the mutation increases CHIKV pseudotypes infectivity in *A. albopictus* cells from 2 to 4 fold but has no effect in *A. aegypti* cells. These results demonstrate a direct effect of the mutation in the CHIKV entry process resulting in differences in cellular tropism.

Although both are derived from Singh's original larva isolates (Singh and Pavri, 1967), several reports suggest differences between C6/36 and C7–10 *A. albopictus* subclones indicating that they may represent different tissue types in the adult insect (Condreay and Brown, 1986, 1988; Miller and Brown, 1992, 1993). Thus, it may be that the 226V change confers a selective advantage in multiple cell types within *A. Albopictus*. Despite the demonstrated fitness advantage of E1 226V virus in *A. albopictus* mosquitoes, almost indistinguishable growth kinetics of CHIKV 226A and 226V viruses in *A. albopictus* C6/36 cells have been described (Tsetsarkin et al., 2007). While this appears to be somewhat contradictory to our results that show that the E1 226V CHIKV pseudotype entry in *A. albopictus* cells C6/36 and C7–10 is subtly more efficient than the wild type, multiple rounds of replication and post-entry steps of the virus life cycle likely conceal this difference and result in almost equivalent growth kinetics. A putative role for E1 226 position in membrane fusion is consistent with its predicted location in the ij loop, a region believed to interact with the target membrane, in contact with the fusion peptide (Schuffenecker et al., 2006). However, we cannot rule out additional implications of the E1 A226V mutation in other steps of the virus life cycle like virus budding and exit. Whether this more efficient entry of the virus is enough to account for the fitness advantage of the E1 226V mutant in *A. albopictus* mosquitoes and its possible association with cholesterol dependence requires further investigation. CHIKV pseudotypes will facilitate the study of the effect of cholesterol in virus entry independently of the post-entry steps.

Our results suggest that C-type lectins act as CHIKV attachment factors and increase infectivity. DC-SIGN and DC-SIGNR lectins bind virus envelope proteins in a carbohydrate-dependent manner, and are likely recognizing high mannose moieties on the viral gps, which contain at least four predicted N-linked glycosylations (asn-X-ser/thr, where X can be any aa apart from proline; Hubbard and Ivatt, 1981). In mouse models of CHIKV infection the liver represents a strong initial target for viral replication, followed by detection of virus in muscle and skin (Couderc et al., 2008). Consistent with this, CHIKV pseudotypes efficiently infect (1.9×10^5 FIU/ml) Huh7 human hepatic cell line. The expression of DC-SIGN and DC-SIGNR, as well as other C-type lectins, in the liver (Lai et al., 2006; Pohlmann et al., 2001) may thus be involved in the early targeting of this organ, while C-type lectins on macrophages and dendritic cells may be involved in disseminating the virus to other tissues.

In this study, we have successfully established retrovirus and rhabdovirus-based pseudotype reporter assays for CHIKV, which to our knowledge, are the first viral reporter systems described for CHIKV. Our results indicate that CHIKV pseudotypes accurately mimic the entry mechanism of the virus and offer a stable and safe source of native antigen thus providing an ideal system for CHIKV immune response analysis, glycoproteins function determination, entry studies and receptor identification. Moreover we have established that mutations in E1 glycoprotein affecting CHIKV entry efficiency mediate alterations in cellular tropism.

Materials and methods

Cells and cell culture

Adherent mammalian cell lines were cultured in DMEM and non-adherent cells were cultured in RPMI, in both cases media was supplemented with 10% FBS and penicillin/streptomycin (15 U/ml). Human endothelial cells (Clonetics) were cultured in Clonetics endothelial cell basal media and human keratinocytes (Clonetics) were cultured in Clonetics keratinocyte cell basal media-2. Human primary blood lymphocytes (PBLs) were isolated as previously described (Simmons et al., 1995) and stimulated in RPMI 1640 containing 20% FCS and 0.5 μg/ml phytohaemagglutinin (PHA, Roche). After 3 days of culture, the cells were spun and resuspended in RPMI

1640 containing 20% FCS and 20 U/ml human interleukin-2 (IL-2, Roche). Human primary monocytes were isolated from donors by apheresis and elutriation. Human primary monocyte-derived macrophages were isolated as previously described (Simmons et al., 1995) and cultured in RPMI supplemented with 10% human serum and penicillin/streptomycin (15 U/ml). The C7–10 *A. albopictus* cell line was kindly provided by Victor Stollar (UMDNJ), while C6/36 were obtained from the ATCC. C7–10 and C6/36 were cultured in MEM supplemented with 10% FBS and penicillin/streptomycin (15 U/ml) while *A. aegypti* cells (ATCC CCL-125) were cultured in MEM supplemented with 20% FBS and penicillin/streptomycin (15 U/ml).

Fetal muscle cells were obtained from elective abortions with the approval of the Committee for Human Research at the University of California, San Francisco and consent of the women undergoing the procedure. The gestational age of the tissues was estimated based on the foot-length of the fetus and ranged from 19 to 24 weeks. Fetal tissues were harvested shortly after termination of the pregnancy and transported to the laboratory on ice in Hank's balanced salt solution with calcium and magnesium (HBSS) and supplemented with 100 μg/ml gentamicin and 2.5 μg/ml amphotericin B (Invitrogen, Carlsbad, CA). Muscle cells were isolated from tongue or thigh muscles and grown similarly to a previously described method (Ozeki et al., 2006). Briefly, the tissue was washed with HBSS solution and, in the case of thigh muscles, the skin removed and the muscle tissue separated from the bone. The tissues were minced using scissors into pieces ≤0.5 cm^3. These fragments were digested using a combination of 1% collagenase class D, 2.4 U/ml grade II Dispase and 0.005% DNase (Roche Diagnostic Corporation, Indianapolis, IN) in 20 ml of HBSS for 30–45 min at 37 °C with gentle agitation. The cell suspension was then passed through a fine wire mesh and any remaining larger fragments disrupted using a glass pestle. The resulting cell suspension was then passed through a 70 m cell strainer (BD Biosciences) to remove any remaining tissue fragments. Cells were washed twice by centrifugation. Muscle cells were cultured on Laminin-1 coated dishes (BD Falcon) in F-10 medium (Invitrogen) supplemented with 20% FBS, 2 μg/ml recombinant human insulin (Roche Diagnostic Corporation), 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Staining of cultured muscle cells was performed with CD146-PE (clone 128018, R&D Systems). CD146 is a marker of fetal myoblasts (Cerletti et al., 2006).

For the 293T-REx cell lines, DC-SIGN and DC-SIGNR were expressed under a tet repressor (Invitrogen) and grown in DMEM medium containing 10% FBS, Zeocin (50 μg/ml), and blasticidin (2.5 μg/ml) as described (Pohlmann et al., 2001). The 293T-REx parental cells were maintained in DMEM medium containing 10% FBS and blasticidin (2.5 μg/ml). Lectin expression was induced by addition of 0.1 μg/ml of doxycycline to the medium.

Both true THP-1 cells, a monocyte cell line (ATCC TIB-202), and B-THP-1 cells were used. B-THP-1 have been shown to be a B cell line, probably derived from Raji cells, that were wrongly classified as THP-1 cells (Wu et al., 2004).

Plasmids

Human codon optimized CHIKV E3/E2/E1 was synthesized (Integrated DNA Technologies, Coralville, IA) based on the structural polyprotein sequence for the S27 African prototype strain of CHIKV (Khan et al., 2002). Assuming an amino-acid numeration of 1 for the initiating methionine of capsid protein, an expression cassette for E3/E2/E1 was synthesized consisting of amino acids 262 to 1248, together with an initiating ATG and upstream Kozak sequence. This was subcloned into the mammalian expression plasmid, pCAGGS (Niwa et al., 1991) using SacI and NheI. A naturally occurring A226V mutation in E1 was introduced using Quikchange site directed mutagenesis (Stratagene) in pUC57 CHIKV E3/E2/E1. A 750 bp fragment carrying the change was swapped into pCAGGS CHIKV E3/E2/E1 and confirmed by sequencing. Plasmids expressing Ebola Zaire

(EboZ), vesicular stomatitis virus (VSV), and amphotropic murine leukemia virus (MLV-A) glycoproteins have been described (Simmons et al., 2002).

Pseudotype production

Lentiviral pseudotypes were produced essentially as described (Simmons et al., 2004) by using 10 μg of luciferase, GFP, or murine CD24 vector (pNL-luc, pNL-gfp, or pNL-HSA based on pNL3-4-R-E-) (Connor et al., 1995) and 30 μg of plasmid-encoding viral envelope. Pseudotyped VSVs were produced essentially as described by Matsuura et al. (2001) by transfecting 293T cells with 16 μg of plasmid-encoding viral envelope and then infecting the cells with recombinant VSVΔG*-G kindly provided by Dr. Whitt (University of Tennessee) at 0.1 multiplicity. If required, virions were concentrated by ultracentrifuge concentration at 28,000 rpm in a SW28 rotor (Beckman) through a 20% sucrose cushion for 1.5 h at 4 °C. The pellets were resuspended overnight in HBSS at 4 °C.

Pseudovirion infection

After p24 ELISA (Aalto BioReagents, Dublin, Ireland) analysis, lentiviral pseudovirions were normalized to contain an equal number of viral particles. Cells were seeded at 2.5×10^4 in 48-well plates 24 h prior to infection. Appropriate amounts of viral supernatant in 200 μl of medium were added to the plates prior to spin infection (Simmons et al., 2002) for 1.5 h at 2100 ×*g* at room temperature. After a 2 h incubation at 37 °C, 300 μl of fresh medium was added and cells were incubated for 72 h. Virus infection was analyzed by measuring the number of GFP expressing cells either by counting green cells using fluorescence microscopy or by flow cytometric analysis. For VSV pseudotype infection mosquito cells were seeded at 1×10^5 cells in 48-well plates 24 h prior to infection. Appropriate amounts of viral supernatant in 200 μl of medium were added to the plates and incubated for 24 h at 28 °C. Virus infection was analyzed by measuring the number of GFP expressing cells by flow cytometric analysis.

Neutralization of pseudovirions was performed by using sera from CHIKV infected individuals (kindly supplied by Azzedine Assal, EFS Centre-Atlantique, France and Robert S. Lanciotti, Centers for Disease Control and Prevention, Fort Collins). Luc pseudovirions were pretitrated on 293T cells to ensure similar input levels of viruses. Sera was inactivated at 56 °C, serially diluted and mixed with luc reporter pseudovirions at a 1:1 ratio and incubated for 45 min at room temperature before addition of 50 μl to RD cells seeded at 2×10^4 cells per well in 96-well plates. After spin infection for 1 h at 1600 ×*g*, cells were incubated for 2 h at 37 °C, washed twice and incubated for 40 h. Luciferase activity was assayed from cell lysates as per manufacturer's instructions (Promega).

Immunological detection of CHIKV envelope glycoproteins

293T cells were transfected as for pseudotype production. The cells were lysed 40 h post-transfection using RIPA buffer, the cell lysate sonicated to shear the DNA and then centrifuged at 2500 ×*g* for 20 min at 4 °C to pellet cell debris. The supernatant was collected and concentrated as described for pseudotype production. Sample buffer and reducing agent were added to the cell lysates and concentrated pseudovirions and the samples were the analyzed by SDS-PAGE using an 8% Bis–Tris gel (Invitrogen). The separated proteins were transferred to a nitrocellulose membrane and blocked with 2% powdered milk in TBS for 30 min at room temperature. The membrane was then incubated in TBS-0.05% Tween-20 with a 1:200 dilution of a mix of heat-inactivated sera from CHIKV infected individuals for 1 h at room temperature. After 3 washes, 10 min each, with TBS-0.05% Tween-20 the membrane was incubated with 1:200 dilution of Alexa 488 goat anti-human antibody (Invitrogen) for 1 h at room

temperature. The membrane was then washed with TBS-0.05% Tween-20 three times for 10 min each, and immunoreactive proteins were visualized using a Molecular Dynamics Storm 860 imaging system in blue fluorescence mode.

Lysosomotropic agents

Cells were incubated with serial dilutions of bafilomicin A, chloroquine, or NH_4Cl (Sigma) 1 h before and during spin infection with Luc pseudovirions. The agent was included in the medium for 5 h after infection, before replacement with fresh medium and assaying for transduction 70 h after infection.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.07.013.

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