Modifications of the PSAP region of the matrix protein lead to attenuation of vesicular stomatitis virus \textit{in vitro} and \textit{in vivo}

Takashi Irie,† Elena Carnero,2 Atsushi Okumura,1 Adolfo García-Sastre2 and Ronald N. Harty1

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St, Philadelphia, PA 19104, USA

Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York City, NY 10029-6574, USA

Correspondence
Ronald N. Harty
rharty@vet.upenn.edu

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The matrix (M) protein of vesicular stomatitis virus (VSV) is a multi-functional protein involved in virus assembly, budding and pathogenesis. The 24PPPY27 late (L) domain of the M protein plays a key role in virus budding, whereas amino acids downstream of the PPPY motif contribute to host protein shut-off and pathogenesis. Using a panel of 37PSAP40 recombinant viruses, it has been demonstrated previously that the PSAP region of M does not possess L-domain activity similar to that of PPPY in BHK-21 cells. This study reports the unanticipated finding that these PSAP recombinants were attenuated in cell culture and in mice compared with control viruses. Indeed, PSAP recombinant viruses exhibited a small-plaque phenotype, reduced CPE, reduced levels of activated caspase-3, enhanced production of IFN-β and reduced titres in the lungs and brains of infected mice. In particular, recombinant virus M6PYA4-R34E was the most severely attenuated, exhibiting little or no CPE in cell culture and undetectable titres in the lungs and brains of infected mice. These findings indicate an important role for the PSAP region (aa 33–44) of the M protein in the pathology of VSV infection and may have implications for the development of VSV as a vaccine and/or oncolytic vector.

INTRODUCTION

Vesicular stomatitis virus (VSV), the prototype of the family \textit{Rhabdoviridae}, has a negative-strand RNA genome of 11 161 nt encoding five structural proteins: the nucleoprotein, phosphoprotein, matrix (M) protein, glycoprotein and large protein. The M protein is a multi-functional protein involved not only in virus assembly and budding, but also in viral pathogenicity (Black & Lyles, 1992; Chong & Rose, 1993; Connor \textit{et al.}, 2006; Craven \textit{et al.}, 1999; Desforges \textit{et al.}, 2001; Ferran & Lucas-Lenard, 1997; Harty \textit{et al.}, 1999; Irie & Harty, 2005; Jayakar & Whitt, 2002; Jayakar \textit{et al.}, 2004, 2000; Kopecky & Lyles, 2003a; Kopecky \textit{et al.}, 2001; Li \textit{et al.}, 1993; Lyles, 2000; Publicover \textit{et al.}, 2006). For example, expression of the M protein alone results in cell rounding, induction of caspase-dependent apoptosis and shut-off of host protein synthesis (Black & Lyles, 1992; Chong & Rose, 1993; Connor \textit{et al.}, 2006; Desforges \textit{et al.}, 2001; Ferran & Lucas-Lenard, 1997; Gaddy & Lyles, 2005; Jayakar & Whitt, 2002; Kopecky & Lyles, 2003a, b; Kopecky \textit{et al.}, 2001; Licata & Harty, 2003; Lyles, 2000; Publicover \textit{et al.}, 2006; Sur \textit{et al.}, 2003). Moreover, expression of the VSV M protein alone in mammalian cells leads to the formation of virus-like particles (VLPs) that are released into the medium (Chong & Rose, 1993; Craven \textit{et al.}, 1999; Harty \textit{et al.}, 1999; Li \textit{et al.}, 1993). A 24PPPY27 type late (L) domain within the M protein has been characterized and shown to be important for efficient budding of VLPs and infectious virus (Craven \textit{et al.}, 1999; Harty \textit{et al.}, 2001, 1999; Irie & Harty, 2005; Irie \textit{et al.}, 2004a, b; Jayakar \textit{et al.}, 2004, 2000).

In our attempts to identify additional L-domain motifs within the M protein, we recovered a panel of VSV recombinants with mutations in or around the 37PSAP40 motif of the M protein. PSAP recombinant viruses have been used previously to demonstrate that the PSAP motif does not possess L-domain activity similar to that of the PPPY motif in BHK-21 cells (Irie \textit{et al.}, 2004a). Interestingly, alterations of amino acids flanking the PSAP core converted this inactive PSAP motif into a functional L domain (Irie & Harty, 2005). For example, the PTAP L domain and flanking residues from human immunodeficiency virus type 1 (HIV-1) p6Gag were able to rescue budding of a PPPY mutant of VSV when inserted at the
The PSAP locus of the VSV M protein (e.g. M6PY>A4 recombinant virus) (Irie & Harty, 2005; Irie et al., 2004a). These data allowed us to conclude that the PSAP region of the M protein was amenable to insertion of heterologous L domains and that the residues flanking the L-domain core motif were important for L-domain activity (Irie & Harty, 2005; Irie et al., 2004a).

As the PSAP recombinants were being characterized for their ability to bud in cell culture, it became apparent that these recombinants were attenuated compared with wt VSV. Here, we report that modifications to the PSAP region of the M protein resulted in reduced levels of CPE in cell culture compared with that induced by wt VSV. The mechanism of attenuation may involve both apoptotic pathways and innate immune responses, as the levels of activated caspase-3 were reduced and levels of beta interferon (IFN-β) were enhanced in cells infected with PSAP recombinant viruses compared with those infected with wt VSV. Lastly, the PSAP recombinant viruses also exhibited an attenuated phenotype following intranasal inoculation of BALB/c mice. Overall, mice infected with the PSAP recombinant viruses did not succumb to infection, and viral titres in the lungs and brains of these animals were reduced significantly compared with those measured in mice infected with a control virus. These findings suggest that the PSAP region (aa 33–44) of the VSV M protein is important for cytopathology and pathogenesis of VSV infection in vitro and in vivo.

**METHODS**

**Cells, viruses and antibodies.** BHK-21 and HeLa cells were maintained in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (all from Life Technologies). All VSV recombinants and a recombinant vaccinia virus (VvT7) were propagated in BHK-21 cells. All virus stocks were titrated by standard plaque assay on BHK-21 cells. Monoclonal antibody (mAb) 23H12 specific for the M protein of VSV was kindly provided by D. S. Lyles (Wake Forest University, School of Medicine, Winston-Salem, NC, USA).

**Construction and recovery of VSV recombinants.** Plasmid pVSV-FL encoding full-length VSV genomic cDNA (Indiana serotype) was kindly provided by J. K. Rose (Yale University, School of Medicine, New Haven, CT, USA). Construction of the PY>A4, M6, M6PY>A4, M6PY>A4-S33M, M6PY>A4-R34E, M6PY>A4-R34A and M6PY>A4-SR>M genes has been described previously (Irie & Harty, 2005; Irie et al., 2004a). All recombinants had the M51R mutation in the M protein (see Fig. 1).

**Measurement of plaque size.** The mean plaque size of each recombinant virus was measured as described previously (Irie & Harty, 2005; Irie et al., 2004a). Briefly, BHK-21 cells in 100 mm dishes were infected to yield approximately 50 plaques per dish. At 24 h post-infection (p.i.), cells were stained with crystal violet and the area of ten plaques on each plate was measured using NIH IMAGE 1.52 software.

**Cell-detachment assay.** BHK-21 cells in six-well plates were infected with recombinant VSVs at an m.o.i. of 10 or inoculated with PBS as a negative control. At the designated time points, cells were fixed with methanol and stained with crystal violet at room temperature for 30 min. Cells were washed with water, the crystal
violet was extracted and the absorbance was measured at a wavelength of 577 nm.

Detection of activated caspase-3. BHK-21 or HeLa cells in six-well plates were infected with recombinant VSVs at an m.o.i. of 5. At the designated time points, cells were harvested by trypsinization, washed with PBS and pelleted by low-speed centrifugation. Each sample was assayed using a Caspase-3/CPP32 Fluorometric Assay kit (BioVision) according to the manufacturer’s instructions.

Pathogenicity in mice. Groups of eight 6-week old female BALB/c mice were inoculated intranasally with $10^7$ p.f.u. of the indicated recombinant virus or with PBS as a negative control. Body weight and survival were monitored every day for 14 days. Three mice were sacrificed at 2 days p.i. and viral titres were measured in the lungs and brain by standard plaque assay on BHK-21 cells. Two identical experiments were performed: Experiment #1 included M51R, PY>A4, M6PY>A4 and M6PY>A4-R34E. Experiment #2 included M6, M6PY>A4-R34A and M6PY>A4-SR>ME.

Detection of IFN-β by ELISA. Human A549 cells in six-well dishes were infected with wt VSV or recombinants at an m.o.i. of 10. Supernatants were harvested at 2, 6 and 12 h p.i. and stored at $-80$ °C. Three samples per time point were assayed in a 96-well format for IFN-β (pg ml$^{-1}$) using the human Interferon ELISA kit (PBL). Virus infections were repeated a total of five times.

RESULTS

Modification of the PSAP region of VSV M affects cytopathology

To study heterologous L domains in the context of a VSV infection, the L domain and flanking residues from p6$^\text{Gag}$ of HIV-1 (SRLEPTAPPEES) were inserted into the PSAP region of the VSV M protein to generate the recombinant virus M6 (Fig. 1a). The M6 recombinant was shown to bud from BHK-21 cells as efficiently as wt VSV (Irie & Harty, 2005). Recombinant M6PY>A4 (Fig. 1a) was also shown to bud from BHK-21 cells as efficiently as wt VSV, indicating that the HIV-1 L domain was functional in the VSV background (Irie & Harty, 2005). Three additional PSAP recombinants were generated containing subtle changes around the PTAP core of the HIV-1 L domain (M6PY>A4-SR>ME, M6PY>A4-R34E and M6PY>A4-R34A; Fig. 1a) (Irie & Harty, 2005). These three recombinants yielded titres slightly lower than (2–5-fold) but comparable to those of M6PY>A4 (Irie & Harty, 2005).

As several of these recombinants had mutations in Met-33 and/or Met-51 of the M protein, immunoprecipitation of infected cell extracts was performed (Fig. 1b) to examine the M protein profile produced by the recombinant viruses. Control viruses and all PSAP recombinants displayed the expected profile for synthesis of M1 (full-length M protein), M2 and/or M3 proteins (Fig. 1b).

Interestingly, all of the PSAP recombinant viruses yielded plaques on BHK-21 cells that were smaller than those produced by wt VSV (Fig. 2). For example, the M6 and M6PY>A4 recombinants yielded plaques approximately 60 % smaller than those of wt VSV and similar in size to those of the budding-defective PY>A4 mutant (Fig. 2). As a comparison, the M51R mutant yielded plaques only 20 % smaller than those of wt VSV (Fig. 2). Lastly, the M6PY>A4-R34E recombinant displayed a fuzzy plaque phenotype, so an accurate plaque size could not be determined (data not shown).

Next, we visually examined the CPE induced by wt VSV or the PSAP recombinants by light microscopy (Fig. 3). BHK-21 cells were mock-infected, or infected with wt VSV, M51R or the indicated PSAP recombinants, and virus-induced CPE was observed at 10 h p.i. (Fig. 3). At this time point, significant CPE was obvious in wt VSV-infected cells and, as expected, CPE was somewhat delayed in M51R-infected cells (Fig. 3). Interestingly, CPE was further delayed in cells infected with the M6 or M6PY>A4 recombinant (Fig. 3). Lastly, virtually no CPE was observed in cells infected with M6PY>A4-R34E at either 10 h (data not shown) or 24 h p.i. (Fig. 3). Thus, these data suggested that the PSAP recombinants were attenuated in their ability to induce CPE in BHK-21 cells. These findings, although unanticipated, were not surprising, as cytopathic activities induced by the VSV M protein have been shown to be genetically separable from the assembly and budding functions of the M protein (Lyles & McKenzie, 1997).

To quantify the level of virus-induced CPE, a cell-detachment assay was performed using infected BHK-21 cells (Fig. 4). Living cells were quantified by measuring the uptake of crystal violet at 4, 6, 8, 10 and 12 h p.i. The most rapid rate of cell detachment (and likely cell death) was observed for M51R- and PY>A4-infected cells (Fig. 4). In contrast, the rate of cell detachment was delayed (beginning at 6 h p.i.) following infection with M6 or M6PY>A4 (Fig. 4). Lastly, the rate of cell detachment was virtually undetectable following infection with the M6PY>A4-R34E recombinant virus (Fig. 4). Taken together, these findings...
indicated that the PSAP recombinants, and particularly the M6PY>A4-R34E recombinant, were attenuated in BHK-21 cells with regard to plaque formation, induction of CPE and rate of cell detachment.

**Detection of activated caspase-3 following virus infection**

To begin to address the mechanism of virus attenuation, we measured the levels of activated caspase-3 in virus-infected cells. VSV is known to induce apoptosis via caspase-3-dependent pathways, and apoptosis is partly responsible for the cytopathology following infection with VSV (Baltzis *et al.*, 2004; Connor *et al.*, 2006; Desforges *et al.*, 2002; Gaddy & Lyles, 2005; Kopecky & Lyles, 2003a, b; Kopecky *et al.*, 2001; Licata & Harty, 2003; Lyles, 2000; Sur *et al.*, 2003). We sought to determine whether the attenuated phenotypes exhibited by the PSAP recombinants in cell culture correlated with defects or delays in the induction of apoptosis. Activated caspase-3 levels were measured at 6 h p.i. in HeLa cells infected with the indicated VSV recombinants (Fig. 5). In this experiment, the level of caspase-3 induced following infection with M51R was set at 1, as all of the PSAP recombinants were created in an M51R background. It should be noted that the caspase-3 activity induced by M51R was approximately 2-fold lower than that induced by wt VSV (data not shown). Interestingly, caspase-3 activity induced by M6 and M6PY>A4 was reduced by more than 50 % compared with that induced by M51R (Fig. 5). Moreover, caspase-3 activity induced by M6PY>A4-R34E was reduced by more than 80 % compared with that induced by M51R (Fig. 5).

As M6, M6PY>A4 and M6PY>A4-R34E recombinants all lack the ability to synthesize the smaller internal M2 and M3 proteins (Jayakar & Whitt, 2002), reduced CPE and caspase-3 levels may reflect the absence of M2 and M3 synthesis. To examine this possibility, the recombinant M6PY>A4-SR>ME was used in this assay. The M6PY>A4-SR>ME recombinant is able to synthesize the M2 protein using Met-33 within the M protein (T. Irie and R. N. Harty, unpublished data). Caspase-3 activity induced by this recombinant, as well as that induced by recombinant virus M6PY>A4-S33M (data not shown), was similar to that induced by M6 and M6PY>A4, but did not reach the level induced by M51R (Fig. 5).

![Fig. 3](image1)

**Fig. 3.** Microscopic analysis of virus-induced CPE. BHK-21 cells were mock-infected or infected with wt VSV, or with M51R, M6, M6PY>A4, or M6PY>A4-R34E virus at an m.o.i. of 3 and cells were observed at 10 h p.i., except where indicated.

![Fig. 4](image2)

**Fig. 4.** Cell-detachment assay using BHK-21 cells. BHK-21 cells were infected with M51R, PY>A4, M6, M6PY>A4, or M6PY>A4-R34E virus at an m.o.i. of 10. The values for cells inoculated with PBS at each time point were set to 1. The data represent the means ± SD of three independent experiments.
It should be noted that we have also examined the time course (6, 10 and 12 h p.i.) of caspase-3 activity induced following infection of HeLa cells with all of the indicated recombinant viruses (T. Irie and R. N. Harty, unpublished data). At 12 h p.i., the levels of activated caspase-3 were virtually identical for wt VSV, M51R, M6 and M6PY>A4. In contrast, even at 12 h post-infection, the level of activated caspase-3 induced by M6PY>A4-R34E was still reduced by approximately 50% compared with the levels of wt VSV and M51R. Taken together, these results suggested that the reduction in CPE correlated with reduced levels of activated caspase-3 in cells infected with the various PSAP recombinants.

**Induction of IFN-β in human A549 cells**

We next sought to determine whether the PSAP recombinants induced levels of IFN-β in infected A549 cells greater than that induced by wt VSV. High levels of IFN-β induced by the PSAP recombinants may contribute to the attenuated phenotype observed in cell culture. Human A549 cells in six-well dishes were infected with wt VSV or the indicated recombinant virus (Fig. 6). Supernatants from infected cells were harvested at 2, 6 or 12 h p.i. Three samples from each time point were then assayed by ELISA for the presence of IFN-β. As expected, infection with wt VSV resulted in inhibition of IFN-β expression, with minimal levels being detected at 2, 6 and 12 h p.i. (Fig. 6; Ahmed et al., 2003; Ferran & Lucas-Lenard, 1997). In contrast, infection with M51R resulted in significantly higher levels of IFN-β, as described previously (Stojdl et al., 2003), than those observed for wt VSV (Fig. 6). The levels of IFN-β induced by M6, M6PY>A4 and M6PY>A4-R34E were also significantly higher than those induced by wt VSV (Fig. 6). IFN-β levels induced by the PSAP recombinants increased with time and the highest levels of IFN-β measured were those induced by M6PY>A4-R34E at 12 h p.i. (Fig. 6). Taken together, these data suggested that attenuation exhibited by the PSAP recombinants correlated with enhanced levels of type-1 IFN produced following virus infection.

**PSAP recombinants are attenuated in mice**

As the PSAP recombinants displayed attenuated phenotypes in cell culture, we sought to determine whether these recombinants were also attenuated in a mouse model of VSV infection. Groups of eight 6-week old BALB/c mice were used to assess survival and virus replication in the lungs and brain. Mice were inoculated intranasally with...
10^7 p.f.u. of the indicated virus, and body weight (data not shown) and survival (Fig. 7) were monitored every day for 14 days. All mice infected with the M51R control virus succumbed to the infection by day 8 p.i. (Fig. 7). In contrast, with the exception of one mouse infected with M6PY>A4-R34A, none of the mice died following infection with the various PSAP recombinants (Fig. 7). Moreover, no appreciable weight loss greater than 20% was observed in mice infected with the PSAP recombinant viruses during the 14 day period (data not shown).

Three mice from each group were sacrificed at 2 days p.i. and viral titres in the lungs and brain were measured by standard plaque assay on BHK-21 cells (Fig. 8). The mean titre of the M51R recombinant in the lungs was 7.4 x 10^6 p.f.u. ml^{-1} (Fig. 8a). In contrast, the titres of M6, M6PY>A4, M6PY>A4-R34A and M6PY>A4-SR>ME were 600–2000-fold lower than that of M51R (Fig. 8). Strikingly, the M6PY>A4-R34E recombinant was unable to replicate in the lungs to the level of detection of this assay (Fig. 8a). It should be noted that the PY>A4 budding-defective mutant was also used to infect mice and that the titre of PY>A4 in the lungs was approximately 40-fold lower than that of M51R (data not shown).

When the brains of infected animals were examined for the presence of virus, only M51R was detected, with a mean titre of 3.2 x 10^3 p.f.u. ml^{-1} (Fig. 8b). None of the PSAP recombinants replicated to detectable levels in the brains of infected animals (Fig. 8b). Taken together, these findings indicated that the PSAP recombinants, in general, were attenuated in mice and that the recombinant M6PY>A4-R34E was the most severely attenuated, both in vitro and in vivo.

**DISCUSSION**

The M protein of VSV plays a role in virus assembly/budding, host protein shut-off and pathogenicity (Black & Lyles, 1992; Chong & Rose, 1993; Connor et al., 2006; Craven et al., 1999; Desforges et al., 2001; Ferran & Lucas-Lenard, 1997; Harty et al., 1999; Irie & Harty, 2005; Jayakar & Whitt, 2002; Jayakar et al., 2004, 2000; Kopecky & Lyles, 2003a; Kopecky et al., 2001; Li et al., 1993; Lyles, 2000; Publicover et al., 2006). These functions are known to be genetically separable (Ahmed et al., 2003; Black et al., 1994, 1993; Lyles & McKenzie, 1997). Thus, whilst assembly/budding (L-domain function) of the PSAP recombinants was similar to that of wt VSV, it was not surprising that the cytopathic activities of these recombinants differed from those induced by wt M protein. We and others have investigated the role of the M protein during virus budding, and a PPPY-type L domain has been identified and characterized (Chong & Rose, 1993; Craven et al., 1999; Harty et al., 2001; Irie et al., 2004b; Jayakar et al.,...
In more recent studies, we found that the PSAP motif downstream of PPPY did not exhibit L-domain activity in BHK-21 cells; however, the PSAP core motif could be converted to an active L domain when the flanking amino acids were replaced with those from HIV-1 p6<sup>268</sup> (Irie & Harty, 2005; Irie et al., 2004a). Indeed, the budding-defective phenotype of the PPPY mutant of VSV was rescued to the budding-efficient phenotype of wt VSV following the insertion of the HIV-1 L domain at the PSAP region of the M protein (Irie & Harty, 2005; Irie et al., 2004a). In addition, efficient budding of PTAP-containing M6 recombinants has been shown to be dependent on expression of host proteins such as tsg101 that interact with the PTAP motif (Irie & Harty, 2005; Irie et al., 2004a).

In this report, the M protein PSAP region modifications attenuate VSV replication for any of the PSAP recombinants was observed (see Fig. 1). Clearly, this single amino acid change had a charged arginine residue (normally present in HIV-1 Gag) rather than the positively charged aspartic acid (normally present in VSV M) and/or oncolytic vector. For example, it has been reported that cellular p53 was involved in the induction of apoptosis in VSV-infected cells, as well as in pathogenicity in VSV-infected mice. Additional experiments are now in progress to identify more precisely the apoptotic pathways affected following infection with these PSAP recombinants.

Of all the PSAP recombinants used, the M6PY>A4-R34E virus was reproducibly the most severely attenuated both in cell culture and in mice. This recombinant contains the PTAP L domain and flanking residues from HIV-1 Gag (Irie & Harty, 2005; Irie et al., 2004a). In contrast, the amino acid change remains unknown, but is of great interest for the potential development of VSV as a vaccine and/or oncolytic vector.

One possibility is that the PSAP mutants of M are defective in their ability to induce apoptosis and thus CPE and cell death. Indeed, much is known regarding the role that the M protein plays in initiating apoptosis (Balachandran et al., 2001; Connor et al., 2006; Desforges et al., 2002; Gaddy & Lyles, 2003a; Kopecky & Lyles, 2003a, 2003b; Licata & Harty, 2003; Lyles, 2000; Sur et al., 2003; Takaoka et al., 2003). For example, Takaoka et al. (2003) reported that cellular p53 was involved in the induction of apoptosis in VSV-infected cells, as well as in pathogenicity in VSV-infected mice. Additional experiments are now in progress to identify more precisely the apoptotic pathways affected following infection with these PSAP recombinants.

A second component of attenuation, particularly in the mouse model of VSV infection, may involve the innate immune response and the induction of type 1 IFN following virus infection. The effect of VSV infection and M protein expression on type 1 IFN production has been well documented (Ahmed et al., 2004, 2003; Balachandran & Barber, 2000; Ferran & Lucas-Lenard, 1997; Takaoka et al., 2003). The M6PY>A4-R34E recombinant induced levels of IFN-β slightly higher than those induced by M51R at both early and late times p.i.; however, overall the levels were comparable (Fig. 6). Low levels of activated caspase-3 observed in cells infected with the PSAP recombinants correlated well with enhanced levels of IFN-β production. These conditions of enhanced cell survival correlate nicely with the attenuated phenotype exhibited by M6PY>A4-R34E, for example.

One interpretation of these findings is that the PSAP recombinants, like M51R, are defective in their ability to shut off IFN-β production. Alternatively, the fact that M51R and M6PY>A4-R34E were dramatically different in their pathogenic potential in mice suggests that significant variations in the innate immune response induced following virus infection in vivo may exist. Experiments to determine the levels of IFN-β in serum and tissues from infected animals, as well as a potential role for TNF in pathogenesis, are currently under way.

In summary, our findings indicated that the PSAP region (aa 33–44) of the VSV M protein is important for inducing cytopathogenicity in cell culture and in mice. Indeed, insertion of a heterologous L domain (from HIV-1 p6<sup>268</sup>) into this region of M resulted in recombinants that could bud to levels similar to wt; however, disruption of this region had dramatic effects on the cytopathic activities of the M protein. Additional experiments will be required to determine more precisely the mechanism(s) of attenuation and whether there are specific relationships between viral L-domain activity and viral pathogenicity. Future studies in this area will be of interest, as the use of VSV as a candidate vector for both gene therapy and selective lysis of...
tumours shows great promise (Ahmed et al., 2004; Balachandran & Barber, 2000; Balachandran et al., 2001; Lichty et al., 2004a, b; Power et al., 2007; Stojdl et al., 2003).

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