

Fusion-active glycoprotein G mediates the cytotoxicity of vesicular stomatitis virus M mutants lacking host shut-off activity

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The cytopathogenicity of vesicular stomatitis virus (VSV) has been attributed mainly to the host shut-off activity of the viral matrix (M) protein, which inhibits both nuclear transcription and nucleocytoplasmic RNA transport, thereby effectively suppressing the synthesis of type I interferon (IFN). The M protein from persistently VSV-infected cells was shown to harbour characteristic amino acid substitutions (M51R, V221F and S226R) implicated in IFN induction. This study demonstrates that infection of human fibroblasts with recombinant VSV containing the M51R substitution resulted in IFN induction, whereas neither the V221F nor the S226R substitution effected an IFN-inducing phenotype. Only when V221F was combined with S226R were the host shut-off activity of the M protein abolished and IFN induced, independently of M51R. The M33A substitution, previously implicated in VSV cytotoxicity, did not affect host shut-off activity. M-mutant VSV containing all four amino acid substitutions retained cytotoxic properties in both Vero cells and IFN-competent primary fibroblasts. Infected-cell death was associated with the formation of giant polynucleated cells, suggesting that the fusion activity of the VSV G protein was involved. Accordingly, M-mutant VSV expressing a fusion-defective G protein or with a deletion of the G gene showed significantly reduced cytotoxic properties and caused long-lasting infections in Vero cells and mouse hippocampal slice cultures. In contrast, a G-deleted VSV expressing wild-type M protein remained cytotoxic. These findings indicate that the host shut-off activity of the M protein dominates VSV cytotoxicity, whilst the fusion-active G protein is mainly responsible for the cytotoxicity remaining with M-mutant VSV.

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INTRODUCTION

Vesicular stomatitis virus (VSV) is a prototype member of the family *Rhabdoviridae*. The virus genome is composed of a non-segmented, single-strand, negative-sense RNA encoding five genes, which are arranged in a module-like, non-overlapping manner. The RNA genome combines with the nucleoprotein N, the phosphoprotein P and the large protein L to form the helical ribonucleoprotein (RNP) complex. This complex is connected to the envelope via the matrix protein M, which binds to both the N protein and the inner leaflet of the lipid-bilayer membrane (Dancho *et al.*, 2009). The M protein plays a key role in VSV morphogenesis and budding (Jayakar *et al.*, 2004).

The VSV envelope contains a single-type transmembrane glycoprotein (G), which has receptor-binding and fusion activities. Following uptake of the virion by receptor-mediated endocytosis and acidification of the endosome, the G protein can perform fusion between the membranes of the viral envelope and the endosome. This results in the release of the RNP complex into the cytosol, where transcription and replication take place. In addition to its role in virus entry, the G protein is important for efficient virus budding and release (Jeetendra *et al.*, 2003).

VSV shows a very broad cell tropism and replicates rapidly in various cell lines that are derived from different species and tissues. Infected cells normally release high numbers of

progeny virus ($>10^9$ p.f.u. ml $^{-1}$) before they ultimately die. The M protein contributes significantly to the virus-induced cytopathic effects (Kopecky & Lyles, 2003) by inhibiting cellular transcription and blocking nucleocytoplasmic RNA transport (Faria *et al.*, 2005; Lyles, 2000; Petersen *et al.*, 2000; von Kobbe *et al.*, 2000). VSV benefits from these activities because the synthesis of type I interferon (IFN) is efficiently suppressed in this way (Ahmed *et al.*, 2003).

Despite the extraordinarily high cytotoxicity of VSV, virus mutants capable of establishing persistent infection in cell culture have been identified (Holland & Villarreal, 1974; Holland *et al.*, 1976; Ramseur & Friedman, 1978; Sekellick & Marcus, 1978, 1979; Youngner & Quagliana, 1976). A number of factors have been implicated in the establishment and maintenance of persistent VSV infections, including the formation of defective interfering particles (DePolo & Holland, 1986), temperature sensitivity (Youngner & Quagliana, 1976) and IFN induction (Nishiyama *et al.*, 1977, 1978; Ramseur & Friedman, 1978). In addition, mutations in the M gene were detected frequently in persistently VSV-infected cells and some of these mutations (e.g. M51R) were shown to abolish the M protein-mediated host shut-off (Ahmed & Lyles, 1997; Desforges *et al.*, 2001). Correspondingly, these mutants do not suppress IFN synthesis and have been proposed as oncolytic viruses for the therapy of tumours that show defects in the type I IFN system (Barber, 2004, 2005; Lichty *et al.*, 2004; Stojdl *et al.*, 2000; Wollmann *et al.*, 2007). Although these VSV mutants expressed M proteins devoid of host shut-off activity, they still revealed cytotoxic features in BHK-21 and HeLa cells, suggesting that other factors also contribute to the induction of apoptosis (Kopecky *et al.*, 2001). Interestingly, VSV expressing wild-type or mutant M protein activates apoptosis through distinct pathways (Gaddy & Lyles, 2005).

In this study, the impact of the M and G proteins on virus-induced cytopathic effects and the induction of type I IFN was analysed by taking advantage of recombinant VSV mutants. Our findings indicate that both the M and G proteins are highly cytotoxic. Consequently, VSV recombinants expressing a mutant M protein along with a fusion-defective G protein or with a deletion of the G gene were able to persist in cells for a prolonged time.

RESULTS

Generation of M-mutant viruses

A series of VSV (serotype Indiana) recombinants was generated by reverse genetics. First of all, a recombinant VSV expressing enhanced GFP from an additional transcription unit located between the G and L genes of the VSV genome was produced (Fig. 1a, VSV*). A number of site-directed mutations were introduced into the M gene of this vector backbone (Fig. 1b). The M33A and M51R substitutions, both implicated in VSV cytotoxicity (Jayakar & Whitt, 2002), were introduced into the M gene either individually or in combination. The M51R substitution was originally identified in the IFN-inducing VSV mutant T1026 (Desforges *et al.*, 2001) and is known to affect M protein-mediated inhibition of host RNA synthesis (Ahmed *et al.*, 2003). Next, VSV recombinants that contained either the single substitutions V221F and S226R or both substitutions together were generated. The double substitution V221F/S226R was previously detected in the VSV mutant TP3, which is able to establish persistent infections in a number of cell lines (Desforges *et al.*, 2001, 2002). V221F/S226R was correlated with reduced viral host shut-off, lower pro-apoptotic activity and induction of type I IFN (Ahmed *et al.*, 2003; Desforges *et al.*, 2002). Finally, all four substitutions M33A, M51R,

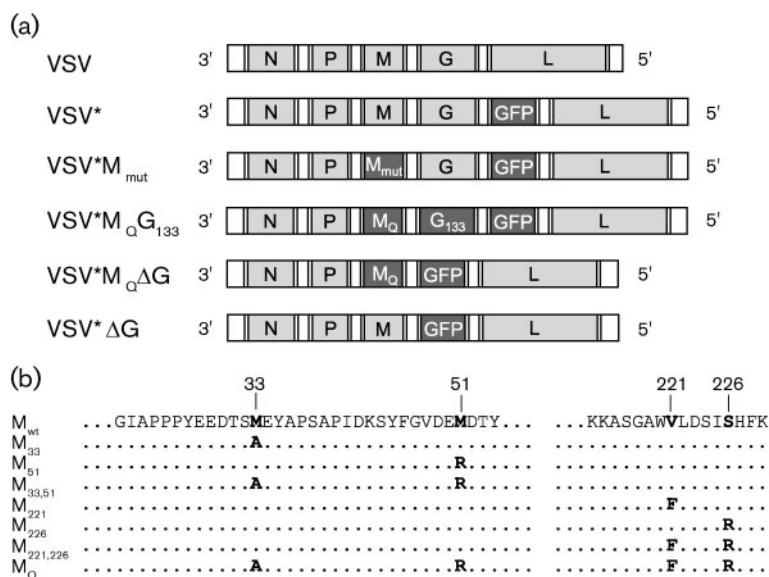


Fig. 1. Genome maps of recombinant VSV. (a) The parental VSV genome encodes the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large RNA polymerase (L). VSV* expresses GFP from an additional transcription unit located between the G and L genes. Several M-mutant recombinants were generated with the amino acid substitutions shown in (b). VSV*M_QG₁₃₃ expresses the quadruple M_Q mutant along with a fusion-defective G protein containing the A133R substitution. In VSV*ΔG and VSV*M_QΔG, the additional transcription unit was deleted and the G gene replaced by GFP.

V221F and S226R were combined in the quadruple mutant VSV* M_Q .

Western blot analysis of cell lysates 6 h post-infection showed that the substitutions had no obvious impact on M protein expression levels (Fig. 2). In addition, the mutations did not change the apparent molecular mass of the M protein, with one notable exception: all mutants containing the M51R substitution migrated in SDS/polyacrylamide gels somewhat faster than the parental M protein. This band shift is typical for the M51R substitution and has been noted previously (Desforges *et al.*, 2001).

Generation of G-mutant viruses

The role of the G protein in VSV-induced cytopathic effects was studied by taking advantage of a fusion-defective G protein. The alanine residue at position 133 is part of the predicted bipartite fusion domain (Roche *et al.*, 2006, 2007) and has been shown to be critical for G protein-mediated membrane fusion (Fredericksen & Whitt, 1995; Sun *et al.*, 2008). Because only fusion activity, but not cell-surface transport, of the G protein was affected when alanine 133 was replaced by arginine (Sun *et al.*, 2008), this substitution was introduced into the VSV* M_Q vector background (Fig. 1a, VSV* M_QG_{133}). Alternatively, the G gene was deleted completely from VSV* and VSV* M_Q , resulting in VSV* ΔG and VSV* $M_Q\Delta G$, respectively. All three mutant viruses were propagated on BHK-G43 cells, a transgenic cell line providing the VSV G protein *in trans* (Hanika *et al.*, 2005). As expected, in the absence of G protein expression, the G-mutant vectors did not produce infectious progeny (Fig. 3c). Western blot analysis verified the absence of G protein in infected cell lysates (Fig. 2).

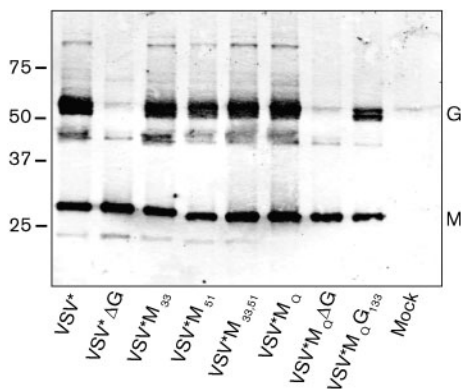


Fig. 2. Western blot analysis of infected BHK-21 cell lysates. At 6 h post-infection with the indicated viruses (m.o.i. of 10), cell lysates were prepared, separated by SDS-PAGE and blotted onto a nitrocellulose membrane. VSV G and M proteins were detected with a polyclonal anti-VSV serum. The positions of molecular mass markers (in kDa) are indicated.

Some M-mutant viruses are attenuated in human fibroblasts

VSV* M_Q replicated in BHK-21 cells with similar kinetics and to similar titres as parental VSV* (Fig. 3a), indicating that the four combined mutations are compatible with the role of the M protein in virus morphogenesis and budding. The replication kinetics of the M-mutant viruses with single or double substitutions did not differ from those of the quadruple mutant (data not shown). In normal human dermal fibroblasts (NHDF), some M-mutant viruses showed a different phenotype. VSV* M_Q replicated to

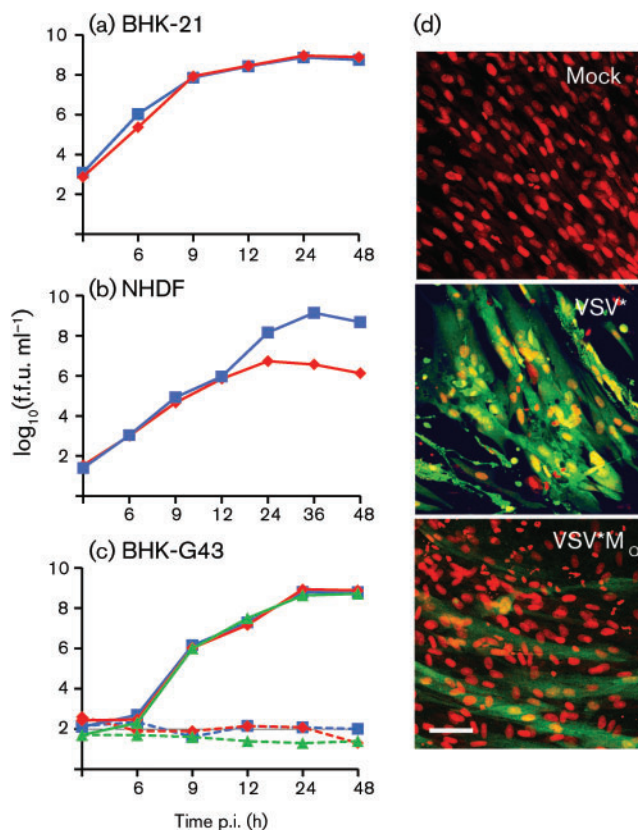


Fig. 3. Replication kinetics of recombinant VSV. (a, b) BHK-21 cells (a) and NHDF (b) were infected with either VSV* (blue lines, ■) or VSV* M_Q (red lines, ◆) using 0.05 infectious units per cell. (c) BHK-G43 cells were treated for 6 h with mifepristone to induce VSV G expression or were left untreated. The cells were infected with VSV* ΔG (blue lines, ■), VSV* $M_Q\Delta G$ (red lines, ◆) or VSV* M_QG_{133} (green lines, ▲) using an m.o.i. of 0.05 and incubated in either the presence (continuous lines) or absence (dotted lines) of mifepristone. At the indicated time points, aliquots of cell-culture supernatant were sampled and titrated in duplicate on BHK-21 cells. Mean virus titres of three infection experiments are shown. (d) NHDF were infected with the indicated viruses (m.o.i. of 0.1). The cells were fixed 24 h post-infection and counterstained with SYTOX Orange (Invitrogen) (red fluorescence). Green fluorescence indicates GFP expression by infected cells. Bar, 75 μ m.

100-fold lower titres than VSV* (Fig. 3b). Fluorescence microscopic analysis of the cells 24 h post-infection (m.o.i. of 0.05) revealed that VSV*M_Q, in contrast to VSV*, did not spread in the cell monolayer beyond the primary infected cells (Fig. 3d). Mutants VSV*M₅₁, VSV*M_{33,51} and VSV*M_{221,226} also showed an attenuated phenotype in NHDF, whereas mutants VSV*M₃₃, VSV*M₂₂₁ and VSV*M₂₂₆ were not attenuated (data not shown).

VSV mutants reveal differences in suppressing cytomegalovirus (CMV) promoter-driven reporter-gene expression

CMV promoter-driven gene expression requires transcription by the nuclear RNA polymerase II and subsequent export of the mRNA into the cytosol, both processes that can be inhibited by the VSV M protein (Ahmed *et al.*, 2003). To analyse the consequences of mutated M and/or G proteins for viral host shut-off activity, BHK-21 cells were transfected with the firefly luciferase gene placed under control of the CMV promoter, and subsequently infected with recombinant virus (m.o.i. of 10). Expression of the reporter gene was abolished completely when the cells were infected with VSV*, VSV*ΔG or VSV*M₃₃, whereas about 50 % relative luciferase activity was detected in VSV*M₅₁-infected cells (Fig. 4). The double mutant VSV*M_{33,51} revealed higher relative reporter activity, but the assay did not allow us to demonstrate the difference between VSV*M₅₁ and VSV*M_{33,51} to be statistically significant ($P=0.054$). The single amino acid substitutions V221F and S226R did not have substantial effects on M protein-mediated host shut-off activity. However, when the two substitutions were combined, statistically significant levels of reporter-gene expression were recorded ($P<0.0001$). The quadruple mutant VSV*M_Q permitted reporter-gene expression levels in the same range as those for the double mutant VSV*M_{33/51}, indicating that the remaining inhibitory activity of VSV*M_{33/51} could not be reduced further by adding the substitutions V221F and S226R. Cells infected with either VSV*M_QG₁₃₃ or VSV*M_QΔG revealed luciferase levels in the same range as those in

VSV*M_Q-infected cells, suggesting that the G protein does not contribute to the inhibition of host protein synthesis.

VSV mutants with compromised host shut-off activity induce type I IFN

The effect of M- and G-mutant viruses on the induction of type I IFN was analysed in NHDF. The magnitude of human IFN-β secretion was quantified by ELISA (Fig. 5). Infection of NHDF with VSV*, VSV*ΔG, VSV*M₃₃, VSV*M₂₂₁ or VSV*M₂₂₆ did not induce statistically significant IFN-β levels ($P>0.05$), whereas about 10 000 pg IFN-β ml⁻¹ was detected following infection with VSV*M₅₁, VSV*M_{33,51}, VSV*M_{221,226} or VSV*M_Q ($P<0.0001$). The G-mutant viruses VSV*M_QΔG and VSV*M_QG₁₃₃ induced IFN-β at lower levels than the propagation-competent VSV*M_Q (about 4000 pg ml⁻¹). Generally, IFN-β was detected readily at 12 h post-infection and did not increase further in the following time. VSV*M_QΔG induced IFN-β more rapidly, with statistically significant amounts already detectable at 6 h post-infection ($P<0.0001$). These findings indicate that only VSV mutants incapable of suppressing CMV promoter-driven reporter-gene expression do induce the synthesis of type I IFN.

VSV*M_QΔG and VSV*M_QG₁₃₃ are compatible with Vero cell survival

At first, the impact of M and/or G gene mutations on VSV-mediated cytotoxicity was studied in Vero cells, which are unable to produce type I IFN (Chew *et al.*, 2009). The viability of the cells in the course of infection was determined by taking advantage of the dehydrogenase activity of metabolically active cells (Cory *et al.*, 1991). Infection of Vero cells with either VSV* or VSV*ΔG (m.o.i. of 10) caused extensive depletion of viable cells within 24 h (Fig. 6a). Microscopic examination of the cells revealed pronounced cell rounding and detachment, resulting in nearly complete destruction of the cell monolayer at 24 h post-infection (Fig. 6c). A rapid decline of cellular viability

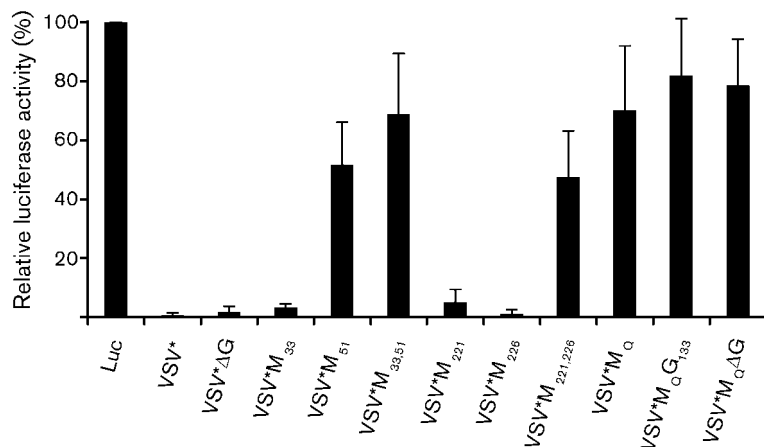


Fig. 4. Analysis of VSV-mediated reporter-gene suppression. BHK-21 cells were transfected with the firefly luciferase gene placed under control of the CMV promoter and subsequently infected with the indicated viruses (m.o.i. of 10). At 6.5 h post-transfection, firefly luciferase activity was determined in cell lysates. The enzyme activity found in mock-infected cells was set to 100%. Mean values and SD of six independent experiments, each run with six replicates, are shown.

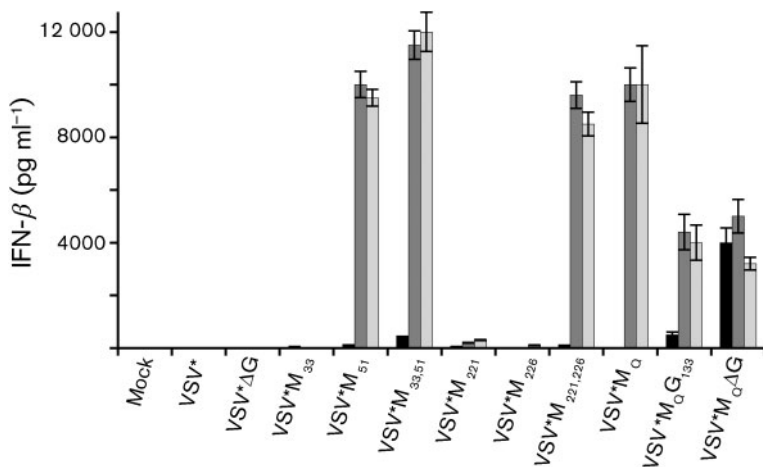


Fig. 5. Induction of IFN- β by M-mutant VSV. NHDF were infected with the indicated viruses (m.o.i. of 10). Cell-culture supernatant was sampled at 6 h (black bars), 12 h (dark-grey bars) and 24 h (light-grey bars) post-infection and tested for the presence of human IFN- β by ELISA. Mean values and SD of three infection experiments are shown.

was also observed following infection with VSV*M₃₃, VSV*M₅₁, VSV*M_{33,51}, VSV*M₂₂₁, VSV*M₂₂₆ and VSV*M_{221,226} (Fig. 6c, and data not shown). In contrast, cell death progressed only moderately when the cells were infected with VSV*M_Q, indicating that the quadruple substitutions reduced, but did not abolish, VSV cytotoxicity. A very different phenotype was observed following infection with either VSV*M_{QΔG} or VSV*M_{QG133}. Most cells not only survived infection with these viruses, but also continued to propagate, as indicated by increasing formazan production (Fig. 6a). Fluorescence microscopic analysis revealed that all cells were still positive for GFP 3 days post-infection (Fig. 6c).

Cell viability was correlated inversely with the activation of caspase-3/7, a key effector protease of apoptosis (Fig. 6b). The highest caspase-3/7 activity was recorded in Vero cells 24 h post-infection with VSV*. VSV*ΔG and VSV*M_Q showed lower relative caspase-3/7 levels, yet the lowest pro-apoptotic activity was found in cells that had been infected with VSV*M_{QΔG} or VSV*M_{QG133}. Taken together, these results indicate that M-mutant (VSV*M_Q) as well as G-mutant (VSV*ΔG) VSV retained high cytotoxicity in IFN-incompetent cells, whereas M-mutant viruses expressing a fusion-defective G (VSV*M_{QG133}) or with a deletion of G (VSV*M_{QΔG}) were compatible with cell survival and cell division.

VSV*M_{QΔG} and VSV*M_{QG133} show reduced cytotoxicity in human fibroblasts

The cytotoxicity of M- and G-mutant VSV was also analysed in IFN-competent NHDF. Infection with VSV*,

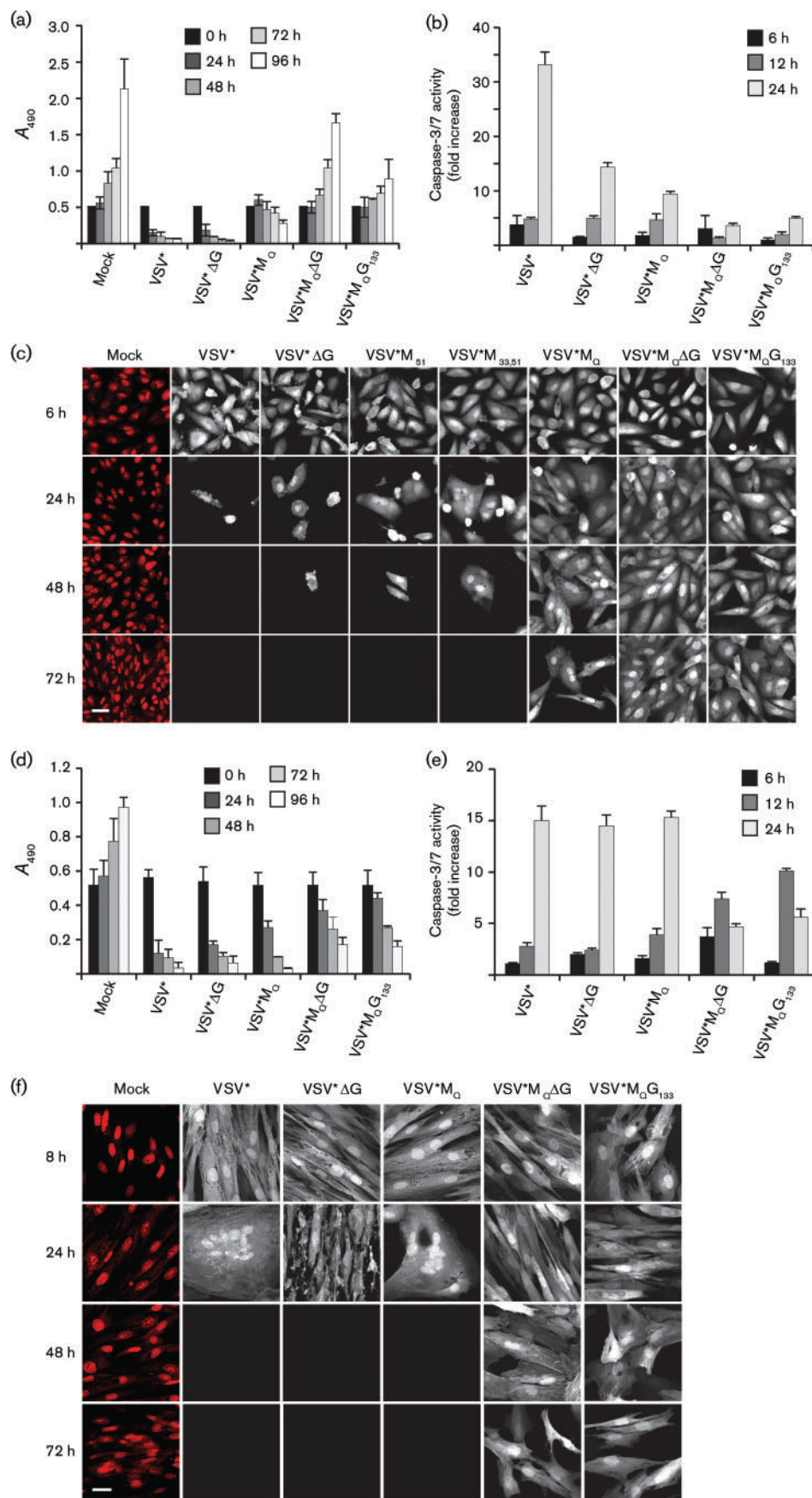
VSV*ΔG or VSV*M_Q (m.o.i. of 10) resulted in a rapid decline of cell viability (Fig. 6d), paralleled by high levels of caspase-3/7 activity (Fig. 6e). Twenty-four hours post-infection of NHDF with either VSV* or M-mutant VSV such as VSV*M_Q, most cells had fused with each other, resulting in the formation of large polykaryons (Fig. 6f). These syncytia were not detected in NHDF following infection with the G-deleted or G-defective viruses. Syncytium formation was less pronounced in infected Vero cells (see Fig. 6c, VSV*M_{33,51}, 24 h).

VSV recombinants with single or double substitutions in the M protein were as cytotoxic for NHDF as the quadruple mutant VSV*M_Q (data not shown). In contrast, when NHDF were infected with either VSV*M_{QΔG} or VSV*M_{QG133}, cell viability also declined, but at a slower rate (Fig. 6d), with several GFP-positive cells still alive 3 days post-infection (Fig. 6f). These two mutant viruses showed statistically significantly lower pro-apoptotic activity than VSV* and VSV*M_Q at 24 h ($P < 0.05$), although caspase-3/7 levels were still higher than those in mock-infected cells (Fig. 6e). In contrast to Vero cells, NHDF stopped propagating when they were infected with either VSV*M_{QΔG} or VSV*M_{QG133}.

Infection of mouse hippocampal slice cultures

VSV was previously shown to cause neurotropic infections in mice, resulting in extensive neurodegeneration (van den Pol *et al.*, 2002). To study the effects of non-transmissible VSV on neuronal cells, hippocampal slice cultures were prepared from normal and IFN (α and β) receptor 1-knockout (IFNAR^{0/0}) mice (van den Broek *et al.*, 1995).

Fig. 6. Analysis of VSV-mediated cytotoxicity. (a, d) Vero cells (a) and NHDF (d) were infected with the indicated viruses (m.o.i. of 10). At the indicated times, the cells were incubated for 60 min at 37 °C with the MTS tetrazolium compound; A_{490} was recorded. Mean values and SD of three infection experiments are shown. (b, e) Caspase-3/7 activity was determined in infected Vero cells (b) and NHDF (e) at the indicated times. The fold increase of caspase-3/7 activity relative to that in mock-infected cells is shown. (c, f) Infected Vero cells (c) and NHDF (f) were fixed at the indicated times and counterstained with SYTOX Orange (Invitrogen). Fluorescence microscopy was performed for detection of GFP (greyscale) and SYTOX Orange (red fluorescence; mock-infected cells only). Bars, 30 μ m.



The slice cultures were infected with either VSV* Δ G or VSV*M_Q Δ G, and GFP expression was monitored for up to 17 days (Fig. 7a). VSV* Δ G revealed a very similar phenotype in infected hippocampal slice cultures from both wild-type and IFNAR^{0/0} mice. High-level GFP expression was detected for 2 days; thereafter, a significant decline of GFP expression was observed, attributable to the cytotoxic properties of VSV* Δ G. Compared with VSV* Δ G, VSV*M_Q Δ G mediated longer-lasting GFP expression in wild-type mouse hippocampus. Nevertheless, GFP expression decreased gradually with time and disappeared at day 12. In IFNAR^{0/0} hippocampus, VSV*M_Q Δ G caused higher GFP expression levels than in wild-type hippocampus, and expression levels decreased only moderately with time (Fig. 7a). This indicates that VSV*M_Q Δ G is able to establish a long-term persistent infection in murine hippocampal cells.

Infection of hippocampal cultures with either VSV* Δ G or VSV*M_Q Δ G resulted in GFP expression in neurons of the pyramidal cell layer (Fig. 7b, c). This was evident from the pronounced labelling of the dendrites of these neurons (Fig. 7b, c) and is consistent with co-labelling of GFP-positive cells with the neuronal marker NeuN (data not shown). We did not observe glial fibrillary acidic protein (GFAP)-positive cells that were positive for GFP (data not shown), further suggesting that mainly neurons are infected.

DISCUSSION

The cytotoxic properties of wild-type VSV have been attributed primarily to the M protein (Desforges *et al.*, 2002; Kopecky *et al.*, 2001), which inhibits both cellular transcription (Ahmed & Lyles, 1998; Black *et al.*, 1993; Yuan *et al.*, 1998) and nucleocytoplasmic mRNA transport (Faria *et al.*, 2005; Her *et al.*, 1997; von Kobbe *et al.*, 2000). The amino acid substitution M51R is known to affect this host shut-off activity (Ahmed *et al.*, 2003; Desforges *et al.*, 2001, 2002; Francoeur *et al.*, 1987), probably by interfering with binding of the M protein to the nuclear-export factor Rael (Faria *et al.*, 2005). In agreement with these previous observations, our recombinant VSV*M₅₁ mutant, but not the parental VSV*, permitted CMV promoter-driven reporter-gene expression and synthesis of type I IFN.

Besides the M51R substitution, the double substitution V221F/S226R, which is located close to the C terminus of the M protein, has been correlated with loss of host shut-off activity and induction of IFN (Ahmed *et al.*, 2003). We found evidence that this phenotype relies on the conjoined substitutions, as neither V221F nor S226R alone allowed CMV promoter-driven reporter-gene expression or IFN synthesis. It is conceivable that V221 and S226 are part of a protein domain that mediates binding of the M protein to a nuclear factor. This interaction might require a certain conformation of the domain, which is only affected if both amino acid positions are mutated conjointly. The crystal

structure of the M protein (Gaudier *et al.*, 2002) suggests that the C-terminal domain is quite close to M51 and is likely to be involved in the same nuclear-pore interactions as those involving M51.

It has been reported previously that the M protein and another viral component contribute to the induction of apoptosis in VSV-infected cells (Kopecky *et al.*, 2001). Moreover, M-mutant or wild-type VSV was found to trigger apoptosis through distinct pathways (Gaddy & Lyles, 2005, 2007). In accordance with these previous findings, we found that Vero cells and NHDF did not survive infection with M-mutant viruses, although host shut-off activity was reduced significantly. Whilst all M-mutant VSV recombinants ultimately killed the cells, cell death progressed at different rates. For example, VSV*M_Q was the least cytotoxic M-mutant virus, which killed the cells at a slower rate than VSV*M_{33,51}. As the host shut-off activities of VSV*M_{33,51} and VSV*M_Q were equally low, this finding suggests that the V221F/S226R substitution may have affected not only host shut-off activity, but also cytotoxic properties of the M protein that are not related to it.

Unlike VSV*M_Q and VSV* Δ G, the VSV*M_Q Δ G mutant showed significantly reduced cytotoxicity ($P < 0.005$) and was able to persist in Vero cells for a prolonged time, indicating that both the M and G proteins contribute to VSV-induced cytotoxic effects. The fusion activity of the G protein is probably responsible for the M protein-independent cytotoxicity, as VSV*M_QG₁₃₃ expressing a fusion-defective G protein could also persist in Vero cells. Indeed, infection with VSV caused the formation of large syncytia in NHDF. The less-pronounced formation of syncytia in Vero cells may explain why VSV*M_Q showed higher cytotoxicity in NHDF than in Vero cells.

Syncytium formation was also observed in the BHK-G43 helper cell line when G protein expression was induced. These and previous findings (Roberts *et al.*, 1999b) indicate that syncytium formation is a more common feature of VSV than thought previously. At first sight, syncytium formation by the VSV G protein is a peculiar phenomenon, because the glycoprotein normally mediates virus entry by a low pH-triggered fusion process. However, previous work suggests that the VSV G protein is an atypical fusion protein that undergoes a reversible conformational change (Roberts *et al.*, 1999b). When the protein is transported to the plasma membrane via the secretory pathway, it encounters the acidic milieu of the Golgi apparatus and undergoes a conformational change. It reaches the plasma membrane in a fusion-active form and therefore is able to perform cell-cell fusion. The resulting syncytia are inevitably doomed to apoptosis, as shown for other fusogenic viruses (Eckardt-Michel *et al.*, 2008; Ferri *et al.*, 2000; Higuchi *et al.*, 2000; Lin *et al.*, 2010).

Our findings are in line with the previous observation that an IFN-inducing phenotype alone is not sufficient to allow persistent VSV infections (Desforges *et al.*, 2001). It may be concluded from our results that persistent infections require that VSV is free not only of host shut-off activity, but also of

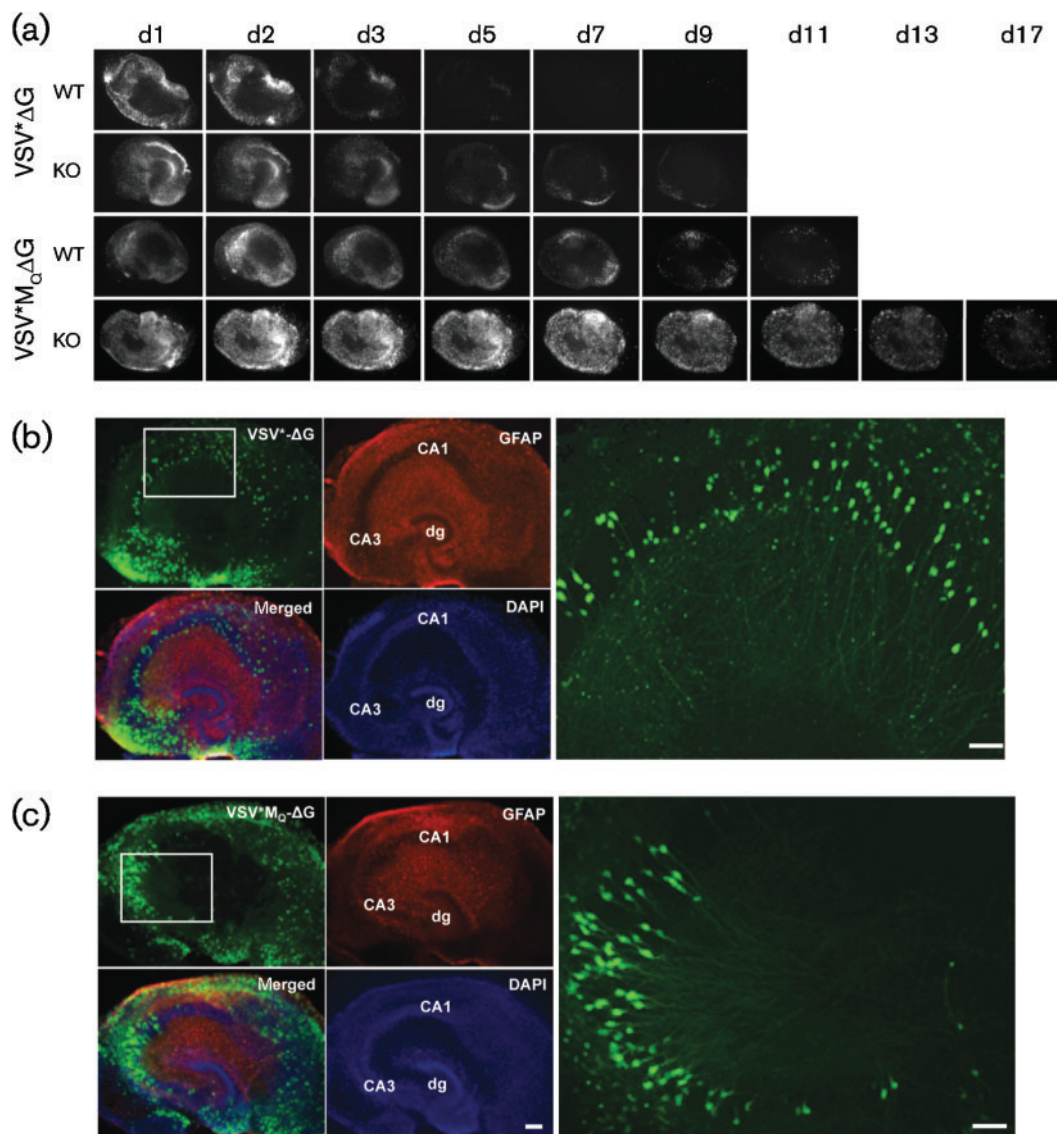


Fig. 7. Infection of mouse hippocampal slice cultures with G-deleted VSV. (a) Wild-type (WT) or $IFNAR^{0/0}$ (KO) mouse hippocampal slice cultures were infected with the indicated vectors. VSV-driven GFP expression was monitored by fluorescence microscopy at daily intervals. (b, c) Immunohistochemical analysis of infected WT hippocampus cultures from 2 days post-infection [see (a)]. Abbreviations: CA1 and CA3, cornu ammonis 1 and 3 of hippocampus; dg, dentate gyros. Right-hand panels represent a magnification of the region indicated in the upper left panel in (b) and (c). Bars, 150 μm (overview images); 60 μm (images at higher magnification).

uncontrolled fusion activity. This may be achieved by mutations directly affecting the fusion activity or the expression levels of G protein. However, the same effect may be accomplished by mutations that lead to reduced genome replication and/or gene transcription. *In vivo*, neutralizing antibodies directed to the G protein may play a role in persistent VSV infections as well (Fultz *et al.*, 1982).

Vero cells not only survived infection with G-deleted or G-defective M-mutant VSV, but also started to proliferate, thereby producing GFP-positive daughter cells. As the virus mutants were defective and unable to produce infectious

progeny, the daughter cells must have received the autonomously replicating viral RNPs in the course of cell division. In contrast, NHDF stopped proliferating when infected with either $VSV^*M_Q\Delta G$ or $VSV^*M_QG_{133}$, although they also survived infection for a prolonged time. This effect may be attributed to the IFN-inducing phenotype of the viruses, as type I IFN is known to exhibit anti-proliferative activities (Platanias, 2005).

Hippocampal slice cultures represent an excellent model to follow the fate of infected cells in a well-organized, IFN-competent tissue. The significant decline of GFP reporter

expression at day 3 post-infection with the G-deleted VSV* Δ G was probably due to the M protein-mediated host shut-off in both wild-type and IFNAR^{0/0} mice. This was contrasted by the ability of the G-deleted M mutant VSV*M_Q Δ G to mediate long-lasting GFP expression in IFNAR^{0/0} hippocampal cells. The lower GFP signals observed following VSV*M_Q Δ G infection of wild-type hippocampus tissue are probably due to the autocrine effects of type I IFN on virus replication and transcription. Although IFN-dependent effects can be excluded in IFNAR^{0/0} hippocampus, a slow decline of GFP expression was also apparent in this tissue 9 days post-infection with VSV*M_Q Δ G. The reason for the decline is not known. On one hand, it is possible that some cytotoxic properties still remain with this virus. On the other hand, the possibility that IFN-independent innate immune mechanisms exist that were able to restrict VSV replication cannot be excluded.

VSV Δ G replicons have been used successfully as experimental vaccine vectors (Kahn *et al.*, 2001; Kalhoro *et al.*, 2009; Kapadia *et al.*, 2008; Majid *et al.*, 2006; Publicover *et al.*, 2005; Roberts *et al.*, 1999a; Schwartz *et al.*, 2007). These replicon vaccines express the wild-type M protein and are highly cytotoxic. In contrast, VSV*M_Q Δ G represents a low-cytotoxic, IFN-inducing RNA replicon that may find some new applications. For example, the induction of type I IFN may prove advantageous for stimulating the immune system, as these cytokines have demonstrated adjuvant activity (Tovey *et al.*, 2008). Moreover, a low-cytotoxic VSV replicon may mediate persistent antigen expression, thereby stimulating 'immune memory'. Finally, the localized synthesis and secretion of type I IFN by VSV*M_Q Δ G may benefit the immunotherapy of certain types of cancer, as type I IFN has anti-proliferative, anti-angiogenic and pro-apoptotic properties (De Palma *et al.*, 2008; Ren *et al.*, 2008).

METHODS

Cells and virus. BHK-21 cells were obtained from the German Cell Culture Collection (DSMZ) and grown in Earle's minimal essential medium (EMEM) supplemented with 5% FBS. BHK-G43 cells were maintained as described previously (Hanika *et al.*, 2005). Vero cells (DSMZ) were cultured with Glasgow minimal essential medium (GMEM) supplemented with 5% FBS. NHDF were purchased from Lonza and maintained in EMEM with 10% FBS for maximum 15 passages. Recombinant modified vaccinia virus Ankara expressing T7 phage RNA polymerase (MVA-T7), a kind gift of Gerd Sutter (Paul-Ehrlich-Institut, Langen, Germany), was propagated and titrated on primary chicken fibroblasts.

Generation of recombinant VSV. The plasmid pVSV*, encoding the full-length anti-genomic (positive-strand) RNA of VSV (serotype Indiana; GenBank accession no. J02428) with an additional transcriptional unit inserted into the G-L intergenic junction, was described previously (Kalhoro *et al.*, 2009). The additional transcription unit of pVSV* drives the expression of GFP. To generate M-mutant VSV, a modified pVSV* lacking any *Bst*EII endonuclease restriction sites was used. This allowed us to replace the M gene of pVSV* by a *Bst*EII restriction sequence, resulting in the plasmid pVSV* Δ M. Wild-type as

well as mutant M genes were inserted into the *Bst*EII site of pVSV* Δ M, resulting in different pVSV*M_{mut} vectors, including pVSV*M_Q (Fig. 1b). The G-deleted genomic plasmids pVSV* Δ G and pVSV*M_Q Δ G were produced from pVSV* and pVSV*M_Q by excising the fragment comprising the VSV G and GFP genes with *Mlu*I and *Nhe*I endonucleases and inserting the GFP gene instead. To generate a fusion-defective VSV, we excised the haemagglutinin (HA) gene from pVSV* Δ G(HA) (Kalhoro *et al.*, 2009) with *Mlu*I and *Bst*EII endonucleases and replaced it with a mutant VSV G gene containing the A133R substitution. This mutation was previously referred to as A117R, due to different numbering of the amino acids in the G protein (Sun *et al.*, 2008). To replace the M gene by M_Q, the *Xba*I/*Mlu*I segment was exchanged for the corresponding region from pVSV*M_Q, resulting in pVSV*M_QG₁₃₃.

Infectious virus was generated from transfected cDNA as described recently (Kalhoro *et al.*, 2009). G-deleted or G-defective VSV vectors were propagated on BHK-G43 cells in the presence of 10⁻⁹ M mifepristone (Sigma). VSV expressing functionally competent G protein was propagated on BHK-21 cells. Infectious virus titres were determined on confluent BHK-21 cells grown in 96-well microtitre plates. The cells were inoculated in duplicate with 40 μ l of serial 10-fold virus dilutions for 1 h at 37 °C. Thereafter, 60 μ l EMEM (in the case of propagation-incompetent VSV) or 200 μ l EMEM containing 0.9% methylcellulose (for propagation-competent VSV) was added to each well, and the cells were incubated for 16 h at 37 °C. Infectious titres were calculated based on the number of GFP-expressing cells or foci per well and expressed as f.f.u. ml⁻¹.

Antibodies. A rabbit anti-VSV serum was kindly provided by Georg Herrler (TiHo Hannover, Germany). The serum was produced by immunizing rabbits with a VSV envelope fraction that was prepared as described by Paternostre *et al.* (1997).

Western blot analysis. Confluent monolayers of BHK-21 cells in six-well plates were infected with recombinant VSV (m.o.i. of 10) and lysed 5 h post-infection with 200 μ l cell lysis buffer (New England Biolabs). The lysate was clarified by centrifugation at 16 000 g for 30 min at 4 °C, supplemented with an equal volume of 2-fold-concentrated SDS sample buffer and heated at 95 °C for 10 min. The lysates (10 μ l) were separated by SDS-PAGE (12% gel) under non-reducing conditions and transferred to nitrocellulose membranes by semi-dry blotting. The blots were incubated successively with primary (rabbit anti-VSV, 1:5000) and secondary (IRDye 680 goat anti-rabbit IgG, 1:10 000) antibodies prior to image acquisition with the Odyssey Infrared Imaging System (LI-COR).

Firefly luciferase gene reporter assay. BHK-21 cells were seeded in 96-well cell-culture plates (2 \times 10⁴ cells per well) and cultured for 24 h at 37 °C. The cells received 50 μ l fresh EMEM/5% FBS per well before they were transfected with 10 μ l medium containing 0.2 μ l Lipofectamine 2000 transfection reagent (Invitrogen) and 0.1 μ g recombinant pCDNA3.1 plasmid (Invitrogen) containing firefly luciferase cDNA. Subsequently, 60 μ l recombinant VSV was added (m.o.i. of 10) and incubated for 6 h at 37 °C. The supernatants were aspirated and the cells were incubated for 30 min at 4 °C with 30 μ l cell lysis buffer (Biotium Inc.). Cell-lysate aliquots (6 μ l) were transferred to white 96-well microtitre plates and luminescence was recorded for 1 s after injection of D-luciferin substrate (Biotium Inc.) with a Centro LB 960 luminometer (Berthold Technologies).

Detection of type I IFN. NHDF were grown in 24-well plates. Confluent cells were infected for 1 h at 37 °C (m.o.i. of 10). The cells were washed twice and incubated with 0.6 ml EMEM/5% FBS at 37 °C. Aliquots of the supernatants were sampled at 6, 12 and 24 h post-infection and stored at -80 °C. Three samples per time point

were assayed for IFN- β in a 96-well format using a Human Interferon ELISA kit (PBL).

Cell-viability assay. Vero cells or NHDF were grown for 24 h at 37 °C in 96-well cell-culture plates (2×10^4 cells per well in 0.2 ml medium and 5% FBS), infected for 1 h at 37 °C with the indicated VSV vectors (m.o.i. of 10) and further cultured with daily changes of medium. At the indicated time points, the cells were incubated for 60 min at 37 °C with 100 μ l PBS and 20 μ l MTS reagent (CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay; Promega). The absorbance (A_{490}) of the aqueous soluble formazan product was determined at 37 °C. Quadruplicate wells were analysed at each time point for the respective virus vector.

Caspase assay. NHDF or Vero cells were grown for 24 h in 96-well plates (2×10^4 cells per well) and infected for 1 h at 37 °C with recombinant VSV (m.o.i. of 10). At the indicated times, the medium was aspirated and the cells were incubated for 10 min at 37 °C with 50 μ l trypsin solution. The cells were suspended with 50 μ l EMEM/5% FBS and 25 μ l of each cell suspension was transferred to white 96-well plates and incubated with 25 μ l Caspase-Glo-3/7 reagent (Promega) for 30 min at room temperature. Luminescence was recorded for 5 s per well using a Centro LB 960 luminometer (Berthold Technologies).

Organotypic slice cultures. Wild-type and transgenic IFNAR^{0/0} mice (Muller *et al.*, 1994) were originally obtained from M. Aguet, Zurich, Switzerland, and kept in the specific-pathogen-free facility at the Institute of Virology, Freiburg, Germany. Hippocampi were dissected from neonate mouse pups (day 1), cut into 400 μ m horizontal sections and maintained as described previously (Mayer *et al.*, 2005). The slice cultures were infected immediately after preparation with 1.5 μ l virus stock, corresponding to about 10^5 f.f.u. For immunofluorescence analysis, slice cultures were fixed with 4% paraformaldehyde and processed as described previously (Mayer *et al.*, 2005). Cellular antigens were stained by incubating free-floating 50 μ m sections with either monoclonal anti-NeuN antibody (Chemikon; MAB377; 1:100) or rabbit polyclonal serum directed against GFAP (Dako; Z0334; 1:500). Alexa Fluor 546-conjugated goat anti-mouse IgG antibody (Molecular Probes; diluted 1:200) and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 111-165-045; diluted 1:400) were used as secondary antibodies. Nuclei were stained with DAPI (Sigma; 1:5000).

Statistical analysis. Statistical analysis was performed using the paired Student's *t*-test, and $P < 0.05$ was considered significant.

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