Inhibition of mRNA export and dimerization of interferon regulatory factor 3 by Theiler's virus leader protein

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Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a neurotropic picornavirus that can persist lifelong in the central nervous system of infected mice, causing a chronic inflammatory demyelinating disease. The leader (L) protein of the virus is an important determinant of viral persistence and has been shown to inhibit transcription of type I interferon (IFN) genes and to cause nucleocytoplasmic redistribution of host proteins. In this study, it was shown that expression of the L protein shuts off synthesis of the reporter proteins green fluorescent protein and firefly luciferase, suggesting that it induces a global shut-off of host protein expression. The L protein did not inhibit transcription or translation of the reporter genes, but blocked cellular mRNA export from the nucleus. This activity correlated with the phosphorylation of nucleoporin 98 (Nup98), an essential component of the nuclear pore complex. In contrast, the data confirmed that the L protein inhibited IFN expression at the transcriptional level, and showed that transcription of other chemokine or cytokine genes was affected by the L protein. This transcriptional inhibition correlated with inhibition of interferon regulatory factor 3 (IRF-3) dimerization. Whether inhibition of IRF-3 dimerization and dysfunction of the nuclear pore complex are related phenomena remains an open question. In vivo, IFN antagonism appears to be an important role of the L protein early in infection, as a virus bearing a mutation in the zinc finger of the L protein replicated as efficiently as the wild-type virus in type I IFN receptor-deficient mice, but had impaired fitness in IFN-competent mice.

INTRODUCTION

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus), a picornavirus in the genus Cardiovirus, is a neurotropic virus with a striking ability to cause persistent infections of the central nervous system (CNS) of the mouse (Theiler & Gard, 1940) (reviewed by Brahic et al., 2005). The genome of TMEV is a single-stranded, positive-sense RNA molecule encoding a long polyprotein, which is processed to yield the mature viral proteins. TMEV encodes two proteins, L and L*, that are not essential for replication of the virus in BHK-21 cells (Kong & Roos, 1991; Kong et al., 1994; Michiels et al., 1997), but that are required for persistent infection of the CNS in vivo (Ghadge et al., 1998; van Pesch et al., 2001; van Eyll & Michiels, 2002; Paul & Michiels, 2006). The L* protein is translated from an additional open reading frame (ORF), overlapping the ORF encoding the L, VP4 and VP2 moieties of the viral polyprotein (Kong & Roos, 1991).

The leader (L) protein of TMEV is a 76 aa protein cleaved from the N-terminal end of the viral polyprotein. It possesses a zinc finger, which has been shown to bind divalent cations (Chen et al., 1995) and to be crucial for L protein function in vitro and in vivo (van Pesch et al., 2001; Delhaye et al., 2004). It has been shown that the L protein can block the production of type I interferons (IFNs) by infected cells and that this inhibition occurs at the transcriptional level (van Pesch et al., 2001; van Pesch & Michiels, 2003). The L protein of TMEV has also been shown to interfere with the nucleocytoplasmic trafficking of cellular proteins in infected cells (Delhaye et al., 2004).
and to trigger the subcellular redistribution of both nuclear and cytoplasmic cellular proteins, most notably interferon regulatory factor-3 (IRF-3).

Encephalomyocarditis virus (EMCV), another member of the genus Cardiovirus, encodes an L protein sharing 35% amino acid identity with the L protein of TMEV. The EMCV and TMEV L proteins share conserved zinc fingers and negatively charged domains. However, the EMCV L protein lacks the C-terminal Ser/Thr-rich domain that is conserved in the L proteins of TMEV strains. In spite of such sequence divergences, the L proteins of EMCV and TMEV share antagonist activities directed against IFN production and nucleocytoplasmic trafficking (Zoll et al., 2002; Lidsky et al., 2006; Paul & Michaels, 2006; Porter et al., 2006; Hato et al., 2007). In the case of EMCV, IFN production inhibition was found to correlate with inhibition of NF-κB activation (Zoll et al., 2002) and suppression of IRF-3 dimerization (Hato et al., 2007). Perturbation of nucleocytoplasmic transport by the EMCV L protein (Lidsky et al., 2006) correlated with the interaction of this protein with Ran GTPase, a crucial regulator of trafficking across the nuclear envelope (Porter et al., 2006). The blockade of macromolecule transport possibly explains the global shut-off of protein expression mediated by the EMCV L protein (Zoll et al., 1996). Recent work suggests that the TMEV L protein also mediates a global shut-off of host-cell protein expression (Baida et al., 2008).

Here, we analysed the possibility that global shut-off and inhibition of IFN gene transcription could result from a global inhibition of cellular transcription. Contrary to this hypothesis, our data showed that the TMEV L protein acts at two different levels in cells. On the one hand, it blocked global protein expression at a post-transcriptional stage, by preventing nuclear export of cellular mRNAs. This activity correlated with nucleoporin 98 (Nup98) hyperphosphorylation. On the other hand, it also acted at the transcriptional level on the expression of selected cytokine and chemokine genes by inhibiting IRF-3 dimerization. In vivo, inhibition of the IFN pathway appears to be an important activity of the L protein at early times of infection.

**METHODS**

**Cells and viruses.** BHK-21, BALB/3T3 and L929 cells were cultured as described previously (van Pesch et al., 2001). TMEV derivatives were produced by electroporation of BHK-21 cells (Michiels et al., 1997) with genomic RNA transcribed in vitro from plasmids carrying the corresponding cDNAs. pTMDA1 carries the genome of virus DA1, a molecular clone of the Daniels strain of TMEV (Daniels et al., 1952; McAllister et al., 1989; Michiels et al., 1997). Virus TMS598 is a DA1 derivative carrying mutations disrupting the zinc finger motif of the L protein (L<sup>6–67</sup> mutation), but which do not modify the amino acid sequence of the L* protein, which is translated from an overlapping ORF (van Pesch et al., 2001). K6 is a DA1 derivative containing capsid mutations adapting the virus to infect L929 cells with high efficiency (Jnaoui & Michaels, 1998). The corresponding L<sup>6–67</sup> mutant was called TM659 (van Pesch et al., 2001). SB3 is another derivative of K6, which carries a deletion encompassing codons 6–67 of the L region (L<sup>6–67</sup>). The pSB3 plasmid, carrying the genome of this virus, was obtained by replacing, in pTM564 (Michiels et al., 1997), an MscI–BamHI restriction fragment in the capsid-coding region with the corresponding fragment of pK6.

**Plasmids construction.** Plasmids were constructed in the pCDNA3 vector backbone (Invivogen), in which gene transcription is under the control of the cytomegalovirus immediate-early promoter (pCMV). A series of plasmids, pTM553, pTM592 and pTM641, were designed to express the L, L<sup>6–67</sup> and L<sup>6–67</sup> proteins, respectively. A second series of plasmids was designed to express a fusion protein comprising the L protein variants and enhanced green fluorescent protein (eGFP) (pCER01, pCER02 and pCER03). pCER03 encodes eGFP alone. In pCER01 (L<sup>6–67</sup>) and pCER02 (L<sup>6–67</sup>), the junction between L and eGFP encompasses codons Pro-75 and Gln-76 of the L protein, a Val-Thr linker and codons Lys-4 and Gly-5 of eGFP. A third series of plasmids was derived from pTM624. This vector contains pCMV, a multi-cloning site and an internal ribosome entry site (IRES) corresponding to nt 392–1065 of TMEV (strain DA1), driving the translation of eGFP (unpublished data). These plasmids co-express the L protein variants and eGFP. The matrix (M) protein-coding region of vesicular stomatitis virus (VSV) was PCR-amplified with primers TM822 and TM823 from cDNA generated from VSV (strain Indiana)-infected L929 cells and cloned in the pTM624 bicistronic construct. Plasmid pCS41 is a pCDNA3 derivative expressing the firefly luciferase (luc) gene (Sommerenaen et al., 2008). Table 1 summarizes the properties of the plasmids used in this study. Primers are presented in Supplementary Table S1 available in JGV Online.

**Capped RNA transcript synthesis.** Capped luciferase mRNA was transcribed in vitro from plasmid pCS41, using T7 RNA polymerase (Roche) and a Ribo m<sup>7</sup>G cap analogue (0.5 mM; Promega), using the conditions recommended by Promega. To eliminate template plasmid DNA, the transcription reaction products were treated with DNase I as described previously (Shaw-Jackson & Michiels, 1999). Residual contaminating DNA amounts, measured by real-time PCR, were not sufficient to generate detectable luciferase activity (data not shown).

**DNA and RNA transfections.** Plasmid DNA was transfected with TransIT-LT1 Transfection Reagent (Mirus) according to the manufacturer’s recommendations. Three microlitres of transfection reagent was used with 1 μg DNA to transfect 3 × 10<sup>5</sup> cells grown in one well of a 24-well plate.

RNA/DNA mixtures were transfected with the TransIT-mRNA Transfection kit (Mirus) into BALB/3T3 cells, at a ratio of 1:9 or 1:39 (100 ng capped RNA transcript and 975 ng plasmid DNA, or 25 ng capped RNA transcript and 975 ng of plasmid DNA). One microlitre of boost reagent was mixed with 1 μl transfection reagent.

**Table 1. Plasmids used in this study**

<table>
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<th>Plasmid properties</th>
<th>L&lt;sup&gt;6–67&lt;/sup&gt;</th>
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<td><strong>Luciferase expression plasmid</strong></td>
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<td>pCMV–Luc</td>
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*Absence of L (L<sup>6–67</sup>) instead of L<sup>6–67</sup>.
and 1 μg nucleic acid in 50 μl Dulbecco’s modified Eagle’s medium. After 15 min of incubation at room temperature, the mixture was added to subconfluent cells grown in 24-well plates.

**Oligo(dT) in situ hybridization (ISH).** BALB/3T3 cells were cultured on 13 mm coverslips. At 50% confluency, the cells were transfected with plasmid DNA expressing the L protein constructs. At chosen times after transfection, the cells were fixed for 8 min with 4% paraformaldehyde in PBS and washed three times in PBS. The ISH protocol was from B. M. Fontoura (Chakraborty et al., 2006). The probe consisted of a 45mer oligo(dT), biotinylated at its 3’ extremity and purified by reverse phase chromatography (Eurogentec). Streptavidin–Cy3 (diluted 1:100; Sigma) was used for detection. Coverslips were mounted in Mowiol 4-88 (Calbiochem), 25% (w/v) glycerol and 0.1% (w/v) diazabicyclooctane (Sigma) in 100 mM Tris-HCl (pH 8.5). The data were analysed by conventional (Leica DM IRB) or confocal (Zeiss Axiovert 135M equipped with a Bio-Rad MRC1024 confocal device) fluorescent microscopy.

**Western blotting for detection of IRF-3 dimerization and Nup98 phosphorylation.** To analyse IRF-3 dimerization, L292 cells were infected for 9 h with KJ6, TM659 or SB3 virus, or were mock infected. Proteins were extracted from the cells under non-denaturing conditions and separated on native polyacrylamide gels as described previously (Hato et al., 2007). For analysis of Nup98, BHK-21 cells were infected for 12 h with KJ6 or TM659 virus, or were mock infected. Proteins were extracted using Tx lysis buffer as described previously (Park et al., 2008). Where indicated, 40 μg protein was treated with 4 U calf alkaline phosphatase (Promega) in the manufacturer’s recommended buffer for 1 h at 37 °C. Samples were analysed by SDS-PAGE followed by Western blotting with a rat monoclonal anti-Nup98 antibody (Sigma-Aldrich).

**Mice and infections.** 129/Sv type I IFN receptor-deficient (IFNAR−/−) mice (Muller et al., 1994) were bred at the animal facility of the University of Louvain, Belgium. 129/Sv mice were purchased from the same source or from Charles River Laboratories. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use. Groups of four (1 and 4 days p.i.) or six (5 days p.i.) 3–4-week-old mice were infected by intracranial injection of 40 μl serum-free medium containing 105 p.f.u. (129/Sv mice) or 2 × 103 p.f.u. (IFNAR−/− mice) of virus. Control mice were injected with 40 μl serum-free culture medium. Mice were anaesthetized with xylazine and ketamine before sacrifice for organ harvest.

**Real-time quantitative RT-PCR.** Total RNA preparations, reverse transcription and real-time quantitative PCRs were performed as described previously (Paul & Michiels, 2006). Real-time PCR standards consisted of 10-fold dilutions of known concentrations of plasmids carrying the sequence to be amplified; pcDNA3-IFN-β (van Pesch et al., 2004) or genomic DNA for IFN-β, pTM410 for TMEV, pTM848 for MCP-1, pTM793 for β-actin, pTM799 for RANTES, pTM844 for interleukin (IL)-6, pTM842 for transforming growth factor (TGF)-β and pCS41 for luciferase. Plasmids pTM793, pTM799, pTM842, pTM844 and pTM849 are derivatives of pCR4-TOPO (Invitrogen) in which the corresponding PCR fragments were cloned. Sense and antisense primers used for IFN-β and MCP-1 amplification were from Petro (2005) and Overbergh et al. (2003), respectively. Primers are presented in Supplementary Table S1.

**RESULTS**

**L protein shuts off synthesis of cellular proteins**

We first confirmed L protein-mediated shut-off of protein expression by testing reporter protein levels in BALB/3T3 cells transfected with plasmids encoding either wild-type or mutant L–eGFP protein fusions or with plasmids encoding bicistronic L–IRES–eGFP constructs. At 24 h post-transfection, cells expressing the Lwt–eGFP fusion showed significantly less fluorescence than cells expressing Lcys–eGFP (5-fold) or LAeGFP (16-fold), in a representative triplicate experiment. The mean fluorescence of cells transfected with the bicistronic plasmid encoding Lwt was also more than 10-fold lower than that of cells that received bicistronic constructs expressing Lcys or LA, confirming inhibition of eGFP expression by the L protein (Fig. 1a).

To detect shut-off at earlier time points after transfection, we used the more sensitive luciferase reporter system. We co-transfected the firefly luciferase expression vector (pCS41) and the L, Lcys or LΔ6–67 expression vector into BALB/3T3 cells and assayed for luciferase activity (Fig. 1b). Luciferase activity measured 7 h after transfection was 12-fold lower in cells expressing L than in cells expressing Lcys or LΔ6–67 (Fig. 1b). Interestingly, luciferase mRNA levels, measured by real-time RT-PCR, varied less than 1.3-fold between cells expressing mutated or wild-type L proteins (Fig. 1c). We concluded that shut-off of luciferase expression by the L protein occurred at a post-transcriptional level.

**L protein does not interfere with cytoplasmic luciferase mRNA translation**

To determine whether the L protein acted at the translational level, capped luciferase mRNA was synthesized in vitro and co-transfected into BALB/3T3 cells along with plasmids encoding the Lwt, Lcys or LΔ6–67 protein. To ensure that cells receiving luciferase mRNA also received the L protein expression plasmid, luciferase mRNA and plasmid DNA were transfected at ratios of 1:9 or 1:39. Luciferase activity was measured at 7, 12 and 14 h post-transfection (Fig. 1d and data not shown). No clear inhibition of luciferase expression by the L protein was observed at any time point in any of four independent experiments involving two productions of capped luciferase mRNA and three different plasmid DNA preparations. In addition, no luciferase activity inhibition occurred in cells that were co-transfected with a 1:9 ratio of mRNA encoding luciferase and L protein, respectively (data not shown). Thus, the L protein of TMEV acted on luciferase expression at a post-transcriptional stage, but upstream of mRNA translation.

**L protein interferes with cellular mRNA export and promotes Nup98 phosphorylation**

Porter et al. (2006) proposed that the L protein of EMCV inhibited mRNA export from the nucleus. To examine the effect of TMEV L protein on mRNA export in a direct manner, we used ISH with an oligo(dT) probe to localize the main pool of poly(A)+ RNA in cells transfected with plasmids expressing the Lwt, Lcys or LΔ6–67 protein or with
the bicistronic plasmids co-expressing these proteins and eGFP (Fig. 2a). As a control, we transfected pCER26, a bicistronic construct that encodes the M protein of VSV, which has been shown to block mRNA export (Her et al., 1997). Non-transfected cells rarely showed nuclear retention of poly(A)$^+$ RNA. In contrast, 17.3% of the cell population transfected with the VSV M-expressing plasmid exhibited a clear nuclear mRNA accumulation. Similarly, 14 and 9.3% of cells transfected with the monocistronic (pTM533) and bicistronic (pTM625) plasmids expressing the L protein, respectively, showed nuclear retention of poly(A)$^+$ RNA (Fig. 2a). In contrast, less than 2% of cells transfected with plasmids expressing the corresponding L protein deletion mutants (pTM641 and pTM624) exhibited a nuclear signal. The cell population transfected with plasmids expressing the L$^{75}$ mutant (pTM592 and pTM626) exhibited a variable and intermediate phenotype.

Although the L protein and, to a lesser extent, the L$^{75}$ mutant inhibited eGFP expression (Fig. 1a), using confocal microscopy it was possible to detect eGFP-positive cells and to evaluate the percentage of cells displaying nuclear poly(A)$^+$ RNA retention among cells that expressed the bicistronic construct (i.e. eGFP-positive cells) (Fig. 2b, c). Cells expressing L$^A$ (pTM624) displayed a mostly cytoplasmic poly(A)$^+$ RNA staining, as typically observed in non-transfected cells. In contrast, nearly 100% of cells expressing the TMEV L protein (pTM625) displayed nuclear accumulation of cellular poly(A)$^+$ RNA, very similar to the results of cells expressing the VSV M protein (pCER26). Surprisingly, 70% of cells expressing the L$^{75}$ mutant (pTM626) showed nuclear accumulation of cellular poly(A)$^+$ RNA, in spite of disruption of the zinc finger in this mutant protein (Fig. 2b, c). This experiment showed that the L protein of TMEV provokes nuclear retention of cellular poly(A)$^+$ mRNA. L protein-mediated nuclear accumulation of mRNA became detectable from 8 h post-transfection and increased with time (data not shown).

The VSV M protein inhibits mRNA export by forming a complex with the transport factor Rae1 and with Nup98 (Faria et al., 2005), a component of the nuclear pore complex thought to play a key role in mRNA export (Powers et al., 1997). To determine whether TMEV L protein might also target Nup98 to inhibit mRNA export, we examined the status of Nup98 in cells infected with TMEV encoding wild-type L or the L$^{75}$ mutant. Fig. 2(d) showed that, at 12 h after infection with wild-type TMEV (KJ6), a slower-migrating form of Nup98 was apparent in cell lysates. This result was probably due to phosphorylation, as treatment with alkaline phosphatase converted...
Nup98 to the single, faster-migrating form. Phosphorylation of Nup98 required a functional L protein, as infection with the Lcys mutant (TM659) had no effect on the migration of Nup98. These results suggested that inhibition of mRNA export by the L protein may be mediated by phosphorylation of Nup98.

**L protein inhibits cytokine and chemokine gene transcription**

The lack of transcriptional inhibition of luciferase mRNA reported here contrasts with the transcriptional inhibition of type I IFN genes reported previously (van Pesch et al., 2001; van Pesch & Michiels, 2003). To confirm IFN gene transcription inhibition and to test whether transcriptional inhibition might involve other genes, we compared the mRNA levels of selected cytokine and chemokine genes in L929 cells infected with virus expressing wild-type or mutated L protein. We studied the transcription of two chemokine genes: RANTES (regulated on activation normal T cell expressed and secreted, or CCL5) and MCP-1 (monocyte chemoattractant protein-1, or CCL2), and of two cytokine genes: IL-6, which is pro-inflammatory, and TGF-β, which is anti-inflammatory. IFN-β was tested as a control to confirm previous data (Fig. 3). Under the conditions used, the viruses infected more than 95% of the cells, as measured by immunohistochemistry (data not shown). At 9 h post-infection (p.i.), gene transcription of
IFN-β, RANTES, IL-6, MCP-1 and TGF-β was upregulated in cells infected with the L<sup>cys</sup> and L<sup>D6–67</sup> mutant viruses, but not in cells infected with KJ6 virus expressing L<sup>wt</sup> (Fig. 3). However, induction levels differed strongly among the genes analysed, being in the ranges of 3000-fold and 300-fold for IFN-β and RANTES mRNA, respectively, and in the ranges of 30-fold and 10-fold for IL-6 and MCP-1 mRNA, respectively. Upregulation of TGF-β transcription was minimal.

These data suggest that, in addition to the post-transcriptional effect of the L protein on mRNA nuclear export, the L protein acts at a transcriptional level on the expression of cytokine and chemokine genes that are typically activated in response to viral infection. The effect was particularly prominent in the case of IFN-β and RANTES genes, whose transcription is strongly dependent on the IRF-3 transcription factor.

**L protein inhibits IRF-3 dimerization**

We next analysed whether L protein-mediated transcriptional inhibition of cytokine and chemokine genes could result from inhibition of IRF-3 activation. IRF-3 dimerization was examined in cells infected with TMEV encoding wild-type and mutant L proteins. As shown in Fig. 4, IRF-3 dimers were detected in cells infected with the L protein mutant viruses (L<sup>cys</sup> or L<sup>D6–67</sup>). In contrast, IRF-3 was almost entirely monomeric in uninfected cells or in cells infected with virus expressing L<sup>wt</sup>. Thus, viral infection can trigger IRF-3 dimerization, but this dimerization is impaired by the L protein.

**Influence of L protein on mouse infection**

To determine the biological relevance of L protein functions and, in particular, the importance of IFN

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**Fig. 3.** Influence of the TMEV L protein on the transcription of cytokine and chemokine genes. L929 cells were infected for 9 h with 2 p.f.u. per cell of KJ6 (L<sup>wt</sup>; filled columns), TM659 (L<sup>cys</sup>; dark shaded columns), or SB3 (L<sup>D6–67</sup>; light shaded columns) virus, or were left uninfected (NI; open columns). mRNA levels, detected by comparative real-time RT-PCR, were normalized against the amount of β-actin in each sample. Histograms show the means ± SD of the relative gene expression. Ratios compared with KJ6-infected samples are shown in the case of viral RNA. Ratios compared with uninfected cells are shown for other genes. Significant differences compared with the corresponding KJ6-infected sample are indicated as * (P<0.05) and ** (P<0.01), determined using an unpaired t-test.

**Fig. 4.** Inhibition of IRF-3 dimerization. L929 cells were infected for 9 h with KJ6 (L), TM659 (L<sup>cys</sup>) or SB3 (L<sup>D6–67</sup>) virus, or were left uninfected (NI). Protein extracts were separated by PAGE under non-denaturing conditions and IRF-3 dimer formation was detected by Western blotting.
production inhibition by the L protein, we compared the replication of wild-type DA1 virus and the L<sup>598</sup> TM598 mutant in the CNS of wild-type and IFNAR<sup>−/−</sup> 129/Sv mice (Fig. 5). As reported previously, the IFNAR<sup>−/−</sup> mice were extremely susceptible to TMEV infection (Fiette et al., 1995). After inoculation of 10<sup>3</sup> p.f.u. DA1 or TM598, IFNAR<sup>−/−</sup> mice rarely survived more than 4 days p.i. At this time point, viral load analysed by RT-PCR in the brain of these mice was between 50- and 100-fold higher than in the brain of wild-type mice, despite the fact that the wild-type mice had been inoculated with a 100-fold higher virus dose (Fig. 5). In IFNAR<sup>−/−</sup> mice, the L<sup>598</sup> mutant virus replicated as efficiently as the wild-type virus.

In contrast, in IFN-competent mice, mutation of the L protein led to a 3.75-fold decrease in virus replication at day 4 p.i. (Fig. 5a, central columns). This replication difference between the L<sup>598</sup> mutant virus and the wild-type virus increased with time and became significant at 5 days p.i. (Fig. 5a, right columns). The fact that the L<sup>598</sup> mutation affected viral yield in wild-type mice but not in IFNAR<sup>−/−</sup> mice suggested that, at least during the first days of infection, the L protein acts to counteract the IFN-α/β response.

We next compared the abundance of IFN-β and RANTES transcripts in the CNS of IFNAR<sup>−/−</sup> mice 1 day after infection with the DA1 and TM598 viruses. IFNAR<sup>−/−</sup> mice were chosen for this analysis to minimize the effect of cell priming by IFN and because the Lcys mutant and wild-type viruses replicated to similar levels in these mice. IFN-β and RANTES transcription was induced in the brains of mice infected by both DA1 and TM598 viruses. As discussed below, IFN-β and RANTES transcripts were only slightly more abundant in mice infected with the L<sup>598</sup> mutant than in mice infected with the wild-type virus (6.5-fold and 2.1-fold, respectively) (Fig. 5b). Thus, the inhibition of cytokine and chemokine gene transcription observed in vitro was less extensive in vivo.

**DISCUSSION**

The L protein of TMEV can trigger a global shut-off of protein expression. Interestingly, this global shut-off, assessed by inhibition of eGFP and luciferase expression, turned out to be neither transcriptional nor translational. Instead, using ISH with an oligo(dT) probe, we showed in a direct manner that the TMEV L protein inhibited export of cellular poly(A)<sup>+</sup> RNA from the nucleus. Such nuclear retention of mRNA could result from L protein-mediated inhibition of Ran GTPase activity (Porter et al., 2006).

Although Ran is not directly involved in mRNA export, Ran inhibition might affect shuttling of proteins such as Dbp5p or TAP/NXF1 (export receptor) or Aly/REF (export adaptor) (Rodriguez et al., 2004) that mediate mRNA export from the nucleus. However, our data revealed that the TMEV L protein mediated phosphorylation of Nup98. This nucleoporin notably interacts with TAP/NXF1 and RAE1, factors involved in mRNA export (Pritchard et al., 1999; Blevins et al., 2003). Thus, our observation that the TMEV L protein mediates phosphorylation of Nup98 suggests another possible mechanism whereby the L protein could inhibit mRNA export by modulating the activity of components of the nuclear pore complex.

We previously reported that type I IFN and RANTES gene expression were inhibited by the TMEV L protein (van...
Pesch et al., 2001; Paul & Michiels, 2006). Here, we showed that expression of other cytokine and chemokine genes, including MCP-1 and IL-6, is also inhibited by the L protein. In contrast to the post-transcriptional shut-off described here, L protein-mediated inhibition of cytokine and chemokine gene expression occurred at the transcriptional level. Transcription of RANTES and IFN-β, which was the most severely affected by the L protein, is known to be upregulated by IRF-3 (Schafer et al., 1998; Lin et al., 1999a). Upon viral infection, IRF-3 is phosphorylated by TBK-1 and IKK-ε kinases. This induces IRF-3 dimerization and translocation into the nucleus (Yoneyama et al., 1998; Lin et al., 1999a, b; Fitzgerald et al., 2003; Sharma et al., 2003) where IRF-3 associates with other transcription factors and CBP/p300 on specific promoter motifs to activate cytokine and chemokine gene transcription (Panne et al., 2007). As recently reported in the case of the EMCV L protein (Hato et al., 2007), the TMEV L protein inhibited IRF-3 dimerization in infected cells (Fig. 4). Intriguingly, IRF-3 dimerization is believed to take place in the cell cytoplasm and to precede nuclear translocation of this factor. Thus, whether inhibition of IRF-3 dimerization can be consequent to nucleocytoplasmic trafficking alteration deserves further investigation.

Mutation of the zinc finger of the L protein affected all of the tested activities of this protein. A possible explanation is that disruption of the zinc finger modified the global fold of the L protein, thus impacting on the different functions of this protein. An alternative explanation is that the various functions of the L protein may be coupled.

It is also interesting to note that not all functions of the L protein were affected equally by the zinc-finger disruption. This mutation had a high impact on cytokine gene transcription inhibition. It also almost fully prevented Nup98 phosphorylation. Yet, it only partially inhibited poly(A)+ RNA export from the nucleus (Fig. 2). This raises the possibility that full inhibition of mRNA export by the L protein is due to disruption of multiple factors in the mRNA export pathway. Alternatively, residual activity of the L99S mutant might go undetected or become apparent according to the experimental settings.

In vivo, IFN-α/β is a key component in the defence against viruses. IFNAR-knockout mice, which are unable to respond to type I IFN, are extremely sensitive to a large panel of viruses and notably to TMEV (Fiette et al., 1995). Here, the importance of the IFN system in the CNS of mice was confirmed by the fact that survival of IFNAR−/− mice was compromised as early as 4 days after intracranial inoculation and that viral load at this time was impressive (more than ten copies of viral RNA per copy of β-actin mRNA in the entire brain). We also tested the importance of the L protein in the antagonism of the IFN response in vivo. In IFN-competent mice, an L99S mutant virus exhibited less fitness than the wild-type virus. In contrast, in the IFNAR−/− mice, the mutant virus replicated at least as well as the wild-type virus (Fig. 5a). The fact that the L99S mutation affected viral yield in wild-type but not in IFNAR−/− mice suggests that, in vivo, antagonism of the IFN system is an important function of the L protein, at least during the first days of infection. However, in contrast to the in vitro situation, inhibition of IFN-β mRNA expression in vivo by the L protein was quite modest. This difference between the in vitro and the in vivo situations may be accounted for by the fact that, in vivo, only a small percentage of the cells is infected. On the one hand, this allows priming of some cells by IFN or by other cytokines before the occurrence of their infection by the virus. Such priming has been shown to override the ability of the L protein to block IFN production (van Pesch & Michiels, 2003). On the other hand, it has been shown previously that, in the CNS of mice infected with TMEV or La Crosse virus, a substantial number of IFN-α/β-producing cells were negative for viral antigen (Delhaye et al., 2006). Recent work notably showed that uptake of picornavirus-infected cells by dendritic cells allows activation of the IFN system (Kramer et al., 2008). Thus, uninfected cells probably detect virus replication products released by infected cells, possibly through Toll-like receptor recognition. As L protein is not expressed in these cells, no inhibition of IFN production would be expected.

Nevertheless, even if IFN production inhibition in vivo is low, the importance of the L protein as an IFN antagonist in vivo is underlined by its influence on virus replication in wild-type compared with IFNAR−/− mice. One might also consider that a small overall reduction in IFN production could hide more extensive local effects that may be physiologically relevant.

In conclusion, as with other viral proteins (such as the NS1 protein of influenza virus, M protein of VSV, 2A protein of poliovirus and L protein of foot-and-mouth disease virus), the L protein of TMEV may be a multi-functional protein devoted to antagonizing the cell and the host innate defences, and in particular the type I IFN response. Two activities have now been identified for the L protein of cardioviruses. Firstly, the L protein represses transcription of virus-induced genes by preventing IRF-3 dimerization. Secondly, by interacting with Ran GTPase (as shown for the EMCV L protein) and/or by promoting phosphorylation of nuclear pore complex proteins (as shown for the TMEV L protein), L proteins would affect nucleocytoplasmic trafficking of proteins and mRNA export, thereby triggering a late global shut-off of protein expression. Whether these two activities of the L protein indeed represent different functions or whether they are connected remains an open question that clearly deserves further investigation.

ACKNOWLEDGEMENTS

We thank Muriel Minet for expert technical assistance. We are grateful to André Tonon for help in FACS analysis, Guy Warnier for help in animal care and Sofia Buonocore for pSB3 construction. We thank Beatriz Fontoura for communication of detailed ISH protocols.
C. R. and B. M. were fellows of the Belgian FNRS (National Fund for Scientific Research). S. D. was the recipient of a FRIA fellowship. S. V. H. is supported by a mosaic grant from the Netherlands Organisation for Scientific Research (NWO-017.002.025). Work in the laboratory of T. M. was supported by the National Fund for Medical Scientific Research (FRSM). Credits aux chercheurs of the FNRS, the FSR of the University of Louvain and the Actions de Recherche Concertées, Communauté Française de Belgique. Work in the laboratory of F. J. M. V. K. was partly supported by grants from the NWO (NWO-VIDI-917.46.303) and the Beijerinck Virology Fund from the Royal Netherlands Academy of Sciences. Work in the laboratory of K. E. G. was supported by ACS RSG109705, NIH AI059467 and AI064432.

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