"The leader protein of Theiler's virus inhibits immediate-early alpha/beta interferon production."

Van Pesch, Vincent ; van Eyll, O ; Michiels, Thomas

ABSTRACT

Theiler's virus is a picornavirus responsible for a persistent infection of the central nervous system of the mouse, leading to a chronic demyelinating disease considered to be a model for multiple sclerosis. The leader (L) protein encoded by Theiler's virus is a 76-amino-acid-long peptide containing a zinc-binding motif. This motif is conserved in the L proteins of all cardioviruses, including encephalomyocarditis virus. The L protein of Theiler's virus was suggested to interfere with the alpha/beta interferon (IFN-alpha/beta) response (W.-P. Kong, G. D. Ghadge, and R. P. Roos, Proc. Natl. Acad. Sci. USA 91:1796-1800, 1994). We show that expression of the L protein indeed inhibits the production of alpha/beta interferon by infected L929 cells. The L protein specifically inhibits the transcription of the IFN-alpha4 and IFN-beta genes, which are known to be activated early in response to viral infection. Mutation of the zinc finger was sufficient to block the anti-interferon activity,...

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Available at: http://hdl.handle.net/2078.1/12943
The Leader Protein of Theiler’s Virus Inhibits Immediate-Early Alpha/Beta Interferon Production

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Received 2 February 2001/Accepted 31 May 2001

Theiler’s virus is a picornavirus responsible for a persistent infection of the central nervous system of the mouse, leading to a chronic demyelinating disease considered to be a model for multiple sclerosis. The leader (L) protein encoded by Theiler’s virus is a 76-amino-acid-long peptide containing a zinc-binding motif. This motif is conserved in the L proteins of all picornaviruses, including encephalomyocarditis virus. The L protein of Theiler’s virus was suggested to interfere with the alpha/beta interferon (IFN-α/β) response (W.-P. Kong, G. D. Ghadge, and R. P. Roos, Proc. Natl. Acad. Sci. USA 91:1796–1800, 1994). We show that expression of the L protein indeed inhibits the production of alpha/beta interferon by infected L929 cells. The L protein specifically inhibits the transcription of the IFN-α4 and IFN-β genes, which are known to be activated early in response to viral infection. Mutation of the zinc finger was sufficient to block the anti-interferon activity, outlining the importance of this motif in the L protein function. In agreement with the anti-interferon role of the L protein, a virus bearing a mutation in the zinc-binding motif was dramatically impaired in its ability to persist in the central nervous system of SJL/J mice.

Theiler’s murine encephalomyelitis virus (TMEV) (or Theiler’s virus), a member of the Picornaviridae family, is a naturally occurring enteric pathogen of the mouse, responsible for central nervous system (CNS) infections (32). The neurovirulent strains (GD7 and FA) cause an acute lethal encephalomyelitis. The persistent strains (DA and BeAn) induce a biphasic disease after intracerebral inoculation of susceptible mice (18). After a mild encephalomyelitis lasting about 2 weeks, mice develop a chronic demyelinating disease, which serves as an experimental model of multiple sclerosis (for review, see references 8 and 25).

TMEV can be recovered from the spinal cord white matter virtually lifelong, indicating that active viral replication occurs during persistence despite the host immune response. Viral persistence appears to be required to induce the chronic demyelinating disease, but the exact mechanisms involved in persistence are still poorly understood. Among the viral determinants of persistence identified, the capsid protein plays a crucial role, probably affecting the tropism of the virus in the CNS (2, 11, 22). However, viral factors allowing the virus to escape the host immune response could also play a pivotal role in establishing persistence.

Antagonism of the innate immune response mediated by alpha/beta interferons (IFNs-α/β) is a common determinant of virulence (33). Indeed, IFNs-α/β are cytokines produced by most cell types in response to viral infection. The antiviral action of IFNs is mediated by the activation of proteins, such as protein kinase R (PKR), the 2’-5’-oligoadenylate synthetase, or the Mx proteins, known to interfere with the viral cycle (29).

The genome of picornaviruses is translated as a long precursor polypeptide that undergoes autoproteolytic cleavage to yield the mature viral proteins. The leader (L) protein of TMEV is a 76-amino-acid-long acidic protein corresponding to the N terminus of the viral polyprotein. L contains a zinc-binding C-H-C-C motif critical for its function in vitro (3, 14). In vivo, the L protein was demonstrated to be essential for neurovirulence of the GDVII strain (1).

In vitro, L is required for viral propagation in L929 cells but not in BHK-21 cells. Since the latter cells are reportedly non-IFN responsive, Kong et al. (14) postulated that the L protein could antagonize the cell interferon response.

The purpose of this study was to test the anti-IFN role of the L peptide and to examine its influence in establishing viral persistence. We show that L inhibits IFN-α/β production and that it selectively blocks the transcription of the immediate-early interferon genes (α4 and β) in L929 cells. The L protein is critical for persistence of the DA1 virus in vivo.

MATERIALS AND METHODS

Construction of mutant viruses. Site-directed mutagenesis (16) was used to introduce three mutations in the region coding for the zinc finger motif of the L peptide of the DA1 persistent strain (Fig. 1) without affecting the amino acid sequence of the L protein encoded by an overlapping reading frame (15). Mutagenesis was performed with oligonucleotide TM56 (5′-AAG GCC TGT GGC AAT GTG GCG CAC ATC TGG GT) on pTM410, a plasmid carrying the L region of the DA1 virus. An Xbal-Van fragment (nt 1 to 1729 of the DA1 virus) was replaced by the corresponding region of plasmid pKJ6, a plasmid carrying the L region of the GDVII strain (18). The resulting plasmid was called TM595.

The capsid coding region of TM595, contained in a BsmBI-BstGI fragment (nt 1265 to 3925 of DA1), was then replaced by the corresponding region of pKJ6 (12), a variant of pTMDA1 with mutations in the capsid coding region that enhance infection of L929 cells. The recombinant carrying the Lmut mutations and...
FIG. 1. Mutations in the zinc-binding motif of the L protein. Point mutations were introduced in codons 11, 12, and 14 of the L-coding sequence of the DA1 and KJ6 viruses to produce the mutant viruses called TM598 and TM659, respectively. Translation of the L protein (L*), and of its mutant form, called Lcys, is shown above the corresponding nucleotide sequence. Nucleotides and amino acids that were mutated are underlined. The amino acid sequence of the L* alternative ORF, shown under the nucleotide sequence, was unaffected by the mutations.

<table>
<thead>
<tr>
<th>Type of encoded L protein</th>
<th>Virus with wild-type capsid</th>
<th>Virus with L29-adapted capsid</th>
</tr>
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<tr>
<td>Wild-type L (Lwt)</td>
<td>DA1</td>
<td>KJ6</td>
</tr>
<tr>
<td>Zinc finger mutations in L (Lcys)</td>
<td>TM598</td>
<td>TM659</td>
</tr>
<tr>
<td>61-amino-acid deletion in L and L* (L27–67)</td>
<td>TM564</td>
<td></td>
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</table>

TABLE 1. Viruses used in the experiments

<table>
<thead>
<tr>
<th>Primers (sense, antisense)</th>
<th>Gene fragment</th>
<th>Fragment length (bp)</th>
<th>No. of cycles</th>
<th>Annealing temp (°C)</th>
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<tbody>
<tr>
<td>TM4, TM132</td>
<td>Virus</td>
<td>1,125</td>
<td>20–30</td>
<td>58</td>
</tr>
<tr>
<td>TM92, TM93</td>
<td>β-Actin</td>
<td>460</td>
<td>20–30</td>
<td>58</td>
</tr>
<tr>
<td>TM235, TM236</td>
<td>Total IFN-α</td>
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<td>30–35</td>
<td>58</td>
</tr>
<tr>
<td>TM237, TM238</td>
<td>IFN-β</td>
<td>354</td>
<td>40</td>
<td>58</td>
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<tr>
<td>TM257, TM258</td>
<td>PKR</td>
<td>680</td>
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<tr>
<td>TM263, TM264</td>
<td>IFN-α4</td>
<td>314</td>
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<td>55–58</td>
</tr>
<tr>
<td>TM265, TM266</td>
<td>IFN-α4-α4</td>
<td>104</td>
<td>35–40</td>
<td>55</td>
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</tbody>
</table>

RESULTS

Construction of L mutant viruses. We constructed a DA1 mutant (called TM598) by disrupting the zinc finger C-H-C-C motif of the TMEV L protein (L27–67) mutant without altering the amino acid sequence of the L* protein encoded by an alternative overlapping open reading frame (Fig. 1). The same mutations were also introduced in the KJ6 virus, which is a

TABLE 2. Primers and PCR conditions

<table>
<thead>
<tr>
<th>Primers and PCR conditions</th>
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<th>No. of cycles</th>
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<td>DA1/KJ6</td>
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<td>β-Actin</td>
<td>460</td>
<td>20–30</td>
<td>58</td>
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<td>TM235, TM236</td>
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<td>IFN-α4-α4</td>
<td>104</td>
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BHK-21 cells. Priming of L929 cells was usually done as follows: 5 x 10⁴ to 1 x 10⁵ cells were incubated in a 24-well plate with 250 μl of pH 2-treated supernatant diluted two times in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. A series of supernatant samples were treated in parallel, with 50 U of a blocking anti-murine IFN-β polyclonal antibody (PBL Biomedical Laboratories) per ml for half an hour at room temperature before priming. At 24 h after priming, cells were infected with KJ6 at an MOI of 1 PFU per cell. Various techniques were used to compare the extent of infection of cells primed with the different pH 2-treated supernatants, including plaque assay, dot blot hybridization, flow cytometry, or immunocytochemistry. The latter techniques involved intracellular labeling of viral antigen with a monoclonal anti-VP1 antibody (F12B3).

IFN activity. IFN activity was quantified by a plaque number reduction assay. BALB/3T3 cells were seeded in six-well plates at a density of 5 x 10⁴ cells per well. After 24 h, cells were treated for 24 h with fourfold serial dilutions of three independent pH 2-treated supernatants (that had been kept frozen at −70°C) or with serial dilutions of reference mouse IFN-β (PBL laboratories), which was itself calibrated against reference IFN from the National Institutes of Health. Cells were then infected for 1 h with a test virus (vesicular stomatitis virus [VSV] or Mengo virus) and overlaid with 0.8% agarose in modified Eagle medium (Gibco-BRL) for plaque assay. Results were confirmed by a standard cytopathic effect reduction assay performed in 96-well plates with the same cells and viruses.

RT-PCR. For the detection of cytokine mRNA, total RNA was extracted from cells by using the Microprop kit (Stratagene). As the IFN genes are intronless, RNA samples were additionally treated with 20 U of fast-protein liquid chromatography-purified DNase I (Amersham Pharmacia Biotech) prior to reverse transcriptase PCR (RT-PCR), as previously described (28). RT-PCRs were performed with and without RT in order to exclude genomic DNA contamination. Conditions used for PCR are presented in Table 2. The sequences of the primers were from reports by Shaw-Jackson and Michalis (28) for β-actin and virus, by Chinsangaram et al. (4) for IFN-β, IFN-α, and PKR, and by Deonarain et al. (6) for IFN-α and IFN-non-α. Note that 1 nucleotide was added to the TM24 primer to increase melting temperature and specificity. Primers sequences were as follows: TM4, TTTCCCTCATCCGGACCTGGT; TM132, GTGCACTATAGAAGAAAAAGCA; TM92, TGGCGCTTTTGACTCAAGGAT; TM93, AGCCCTGGCTGCCTCAAC; TM235, ATGCTACTGTCCTGGTCTCCT; TM236, AGGGCTCTCCAGYTTCTGCTCTG; TM237, CATCAACTATAAGCACTGCTCA; TM238, TTCAAGTGGAGAGCAGTTGAG; TM257, TM258, PKR 680 30–35 58

<table>
<thead>
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<th>No. of cycles</th>
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<td>95–98</td>
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<tr>
<td>104</td>
<td>55</td>
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</tbody>
</table>

Infection of mice. Three-week-old female NIH mice (from IFAA-CREDO) were inoculated intracranially in the right hemisphere with 40 μl of viral suspension containing 10⁵ PFU of the indicated virus. At 5 or 45 days postinfection, groups of four mice were sacrificed and their viral loads in brain and spinal cord were quantified by dot blot analysis. Note that some of the data for DA1- and TM564-infected mice were reported previously (34).
infection. neighboring cells resistant to both wild-type and mutant virus such as IFN-α/β to the low MOI used, the results suggest that a soluble factor, mutant. As coinfection of cells by both viruses was unlikely due of viral RNA was not higher than that in the case of the L protein (TM598) virus. In the case of the mixed infection, the level of 24 and 48 h postinfection (Fig. 2).

As reported by Kong et al. (14), viral replication of the L929 cell infection (14 h) at an MOI of 0.2 PFU per cell, the yield of infectious virus was slightly (2.4 times) higher for the KJ6 virus than for the L39a derivative TM659 (7.5 ± 1.08 × 10^4 and 3.2 ± 0.85 × 10^4 PFU per ml, respectively).

A soluble factor secreted by L929 cells is capable of restricting viral propagation. L929 cell monolayers were infected at an MOI of 0.1 PFU per cell with either the wild-type (DA1) or the L39a mutant (TM598) virus or with a 1:1 mixture of the two viruses. Viral replication was assessed by dot blot hybridization 24 and 48 h postinfection (Fig. 2).

As reported by Kong et al. (14), viral replication of the L-mutant virus was restricted in L929 cells compared to that of the wild-type virus. In the case of the mixed infection, the level of viral RNA was not higher than that in the case of the L mutant. As coinfection of cells by both viruses was unlikely due to the low MOI used, the results suggest that a soluble factor, such as IFN-α/β, secreted by TM598-infected cells rendered neighboring cells resistant to both wild-type and mutant virus infection.

\[ \text{Supernatants from KJ6 (L} \alpha^w) \text{, TM659 (L} \alpha^a) \text{, or mock-infected cells were collected 48 h after infection. These supernatants were then treated at pH 2 to inactivate the virus and then used to prime L929 cells. Cells primed with the various supernatants were then infected by KJ6 to compare their resistance to viral infection.} \]

As shown in Fig. 4A, priming of L929 cells with a supernatant from KJ6-infected cells did not protect cells from subsequent infection better than priming with a supernatant from mock-infected cells. This suggests that little or no IFN-α/β was produced in the supernatant of KJ6-infected cells in the conditions used. In contrast, priming of L929 cells with supernatants from L-mutant-infected cells strongly inhibited subsequent infection (about an 80% reduction in the number of infected cells).

To confirm that the inhibition of viral infection was really due to the presence of IFN-α/β in the pH 2-treated supernatants, samples of pH 2-treated supernatants were treated in parallel with an anti-IFN-α/β antibody prior to cell priming. As shown in Fig. 4B and C, such a treatment completely abolished the antiviral effect of cell priming.

The amount of IFN present in the supernatant of infected cells was quantified using both VSV and the Mengo virus strain of EMCV as reporter viruses. In the conditions used (pH 2-treated supernatants collected 48 h after infection and stored frozen), TM659-infected cells consistently produced between 5 × 10^3 and 20 × 10^3 U of IFN per ml, protective toward both VSV and Mengo virus infections. No IFN activity (<100 U per ml) could be demonstrated in the supernatant of KJ6-infected cells.

In conclusion, this experiment shows that the L peptide is capable of inhibiting IFN-α/β production by L929 cells. Dis-
ruption of the zinc-binding motif of the protein is sufficient to block this anti-IFN activity.

Infection of STAT-1-deficient cells. As IFN-α/β signaling occurs through STAT-1 phosphorylation and translocation, we analyzed whether L mutant viruses could replicate in STAT-1 deficient cells. Therefore, we compared the infection of 2fTGH human fibroblasts and of their STAT-1−/− derivatives (U3A cells) by the DA1 and TM598 viruses. Infections were performed at 0.5 or 5 PFU per cell. Replication was followed at different time points between 14 and 48 h by dot blot hybridization (Fig. 5). U3A cells appeared to be highly susceptible to infection by both the wild-type and Lcys viruses. In these cells, the difference of replication between wild-type and mutant viruses was low (1.1 to 1.6 times higher for the wild type) while, in 2fTGH cells, the difference was more pronounced (3.7 to 8 times higher for the wild type). This observation nicely fits an anti-IFN role for L. One does not know at this time whether the small but reproducible reduction of replication observed in U3A cells for the mutant virus reflects a STAT-1-independent effect of the IFN pathway or an additional role for the L protein.

L protein selectively inhibits transcription of immediate-early IFN genes. In order to examine whether the inhibitory effect of the L protein on the IFN-α/β production was due to transcriptional repression, RT-PCRs were performed to compare IFN mRNA levels in cells infected with the wild-type and mutant viruses. RNA was extracted from L929 cells infected for 7 h with KJ6 or TM659 at an MOI of 5 PFU per cell. At this MOI, the proportion of cells infected by the two viruses was comparable (more than 95% of antigen-positive cells), as measured by fluorescence-activated cell sorter analysis (not shown).

RT-PCR results (Fig. 6) showed a strong inhibition of the transcription of IFN-α4 and IFN-β in KJ6-infected cells 7 h after infection. In contrast, in these cells, the mRNA levels of total IFN-α and of IFN-non-α4 were similar if not higher than those in cells infected with the mutant virus. This specific inhibition of IFN-α4 and IFN-β by the Lα4-expressing virus was confirmed in a time course experiment (1 to 7 h) (Fig. 7) and in several independent experiments with samples analyzed 12 and 24 h after infection at MOIs of 5 and 0.1 PFU/cell (data not shown). No clear effect of the viral infection was seen on the level of PKR mRNA.

L protein is essential for persistence of TMEV in CNS. The L peptide was previously shown to be essential for the neurovirulence of the GDVII virus strain (1). We wanted to determine whether L was also required for the pathogenesis of the persistent strain DA1. We therefore assessed, by dot blot hybridization, the level of viral persistence of wild-type DA1 and mutant TM598 (Lα4) and TM564 (L<sup>Δ7-67</sup>) viruses in brains and spinal cords of SJL/J mice 5 and 45 days postinfection (Fig. 8).

The 61-codon deletion of TM564 had a dramatic effect on viral persistence, as this virus was not detected by RT-PCR of the CNS of the mouse 45 days after infection. However, it is noteworthy that the deletion present in the L region of the virus has no effect on viral persistence in the CNS.
virus also affected the L* reading frame so that the lack of persistence cannot be attributed solely to the mutations in L.

The mutation of L in TM598 also had a strong impact on virus persistence. At 45 days postinoculation, the amount of Lcys-mutant virus RNA in the spinal cord was 36 times lower than that of the wild-type virus. Viral persistence was severely but not completely blocked at this time since viral RNA of the Lcys mutant could still be detected by RT-PCR in the spinal cord of four out of four mice. Sequencing of the RT-PCR products confirmed the virus identity and showed that no revertants were selected during infection. The effect of the L protein zinc-binding motif disruption was already apparent 5 days postinfection (fourfold effect). This could indicate that a functional L peptide is required for efficient viral infection during the acute phase of the disease and is in agreement with the observed anti IFN-α/β role of the protein.

DISCUSSION

Inhibition of IFN-α/β by L peptide. Kong et al. (14) have observed that the L peptide of TMEV is required for viral spread in L929 cells, but not in non-IFN-responsive BHK-21 cells. On the basis of these observations, they proposed that the L protein could interfere with the host IFN response. Our results establish that the L peptide indeed inhibits IFN-α/β production by infected L929 cells, as supernatants of cells infected with a virus expressing the wild-type L protein failed to prime naive cells for viral resistance.

To analyze the level at which repression of IFN production occurred, we performed RT-PCRs to monitor IFN mRNA levels in infected cells. As soon as 7 h after infection, we observed a strong inhibition of the transcription of the IFN-α4 and IFN-β genes in L929 cells infected with the KJ6 virus. This effect is strikingly selective as the transcription of IFN-non-α4 genes and that of the PKR gene were not decreased.

IFN-α4 and IFN-β are termed the immediate-early IFN genes, being the first two subtypes synthesized following viral infection (6, 19, 20, 26). Several transcriptional activators have been shown to cooperate in forming an active enhanceosome at the IFN-β promoter (35). These include IRF-3, NF-κB, and ATF/c-Jun, which are all activated by phosphorylation in the cytoplasm and translocate to the nucleus following viral infection. The immediate-early IFNs are subsequently secreted and act in a paracrine manner to induce an antiviral state in neighboring cells. They might also act in an autocrine fashion to induce the transcription of the other IFN-α subtypes.

The selective inhibition of IFN-α4 and IFN-β observed in L929 cells suggests that the L peptide targets a specific factor involved in their transcription, although at this stage we cannot exclude the possibility of a posttranscriptional effect. IRF-3 is an obvious candidate for interaction with the L peptide, as this factor is known to specifically activate the transcription of IFN-β and IFN-α4 (13). IRF-3 is constitutively present in the cytoplasm of uninfected cells. Viral infection triggers a signaling cascade which leads to the C-terminal phosphorylation of IRF-3 (27), enabling it to homodimerize and to translocate to the nucleus, where it cooperates with CBP/p300 to activate the transcription of the IFN genes (17, 31, 36). Experiments are in progress to determine whether the L peptide interacts with IRF-3.

It is intriguing to note that total IFN mRNA synthesis was activated in KJ6-infected cells in spite of the repression of

FIG. 6. Specific inhibition by the L protein of immediate-early IFNs (α4 and β). Total RNA was extracted from L929 cells infected for 7 h with viruses expressing the wild-type L protein (KJ6) or the Lcys mutant (TM659) or from mock-infected cells (–). RT-PCR was used to measure mRNA levels of total IFN-α, IFN-non-α4, IFN-α4, IFN-β, and PKR. Viral RNA and β-actin mRNA were amplified as controls. PCR conditions used are shown in Table 1. Note the strong inhibition of IFN-α4 and IFN-β, but not of total IFN-α, in cells infected with the wild-type virus, producing the Lwt protein.

FIG. 7. Kinetics of IFN-α4 and IFN-β inhibition. As described in the legend to Fig. 6, except that samples were analyzed from 1 to 7 h after infection to check whether inhibition was already effective at early times of IFN induction.
IFN-β and IFN-α4. Indeed, in current murine models (20, 26), synthesis of the late IFN-α subtypes depends on the transcriptional induction of IRF-7 by immediate-early IFNs. Since immediate-early IFNs were repressed here, one might postulate that, in this case, transcription of some late IFNs subtypes could occur independently of IRF-7 activation.

**L peptide is critical for viral persistence in vivo.** A virus with mutations in the zinc-binding motif of the L peptide is severely impaired in its ability to persist. The L peptide influences infection in the early stages of the infection, as our data show a fourfold reduction of viral RNA for the Lcys mutant virus already 5 days postinfection. These data are in good agreement with previous results from Calenoff et al. (1), who showed that the L peptide is also important for the neurovirulence of the GDVII strain. The fact that L is required early by TMEV in the CNS could be a clue that it is important for the virus to counteract the innate host immunity and, in particular, the IFN-α/β response.

It is clear, however, that IFN inhibition by L is not complete, as the disruption of STAT-1 in U3A cells and of IFN-α/β receptor in knockout mice (10) dramatically enhanced infection by the wild-type virus (expressing L). This might reflect an inability of the virus to completely counteract a potent IFN response of the host. On the other hand, modulation rather than blockade of the IFN response might represent a better strategy to allow viral persistence and favor host-to-host transmission of the virus.

**Conservation of anti-IFN role of picornavirus L.** Although the picornavirus genome organization is rather well conserved, only cardioviruses and aphthoviruses express L. The L protein of aphthoviruses is a protease responsible for host cell protein synthesis shutoff through cleavage of eIF4G, a factor required for translation initiation (7). This protein, which is unrelated to the L protein of cardioviruses, was also found to have anti-IFN activity, possibly through the inhibition of protein synthesis (4).

The Cardiovirus genus includes TMEV and EMCV. As in the case of aphthoviruses, the L protein of Mengo virus (an EMCV strain) was proposed to participate in host cell protein synthesis shutoff and to affect IFN-α/β production by infected cells (37). The L protein of EMCV and TMEV share about 35% of identical amino acids. In spite of this rather low identity, the zinc-binding motif is perfectly conserved in all the strains sequenced so far, suggesting some conserved roles for these proteins. We found that IFN inhibition by the L protein of TMEV was specific for immediate-early IFN and thus unlikely to result merely from host cell translational shutoff. L proteins of cardioviruses might thus interfere with different signal transduction pathways to induce translational shutoff and immediate-early IFN inhibition.

**ACKNOWLEDGMENTS**

We are indebted to Daniel Gonzalez-Dunia and Sylvie Syan (Pasteur Institute, Paris, France) for help with the interferon biological assay. We thank Eliane Meurs (Pasteur Institute, Paris, France) for the gift of YSV and Ann Palmenberg (University of Wisconsin, Madison) for the gift of pMC24. We thank Michel Brahic (Pasteur Institute, Paris, France) for the F12B3 monoclonal antibody and for long-term collaboration. We are grateful to Ian Kerr (Imperial Cancer Research Fund, London, United Kingdom) and his team for rapid sending of...
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