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The Leader Proteinase of Foot-and-Mouth Disease Virus Inhibits the Induction of Beta Interferon mRNA and Blocks the Host Innate Immune Response

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We have previously shown that the leader proteinase (L^{pro}) of foot-and-mouth disease virus (FMDV) blocks cap-dependent mRNA translation and that a genetically engineered FMDV lacking the leader proteinase coding region (A12-LLV2) is attenuated in cell culture and susceptible animals. The attenuated phenotype apparently is a consequence of the inability of A12-LLV2 to block the expression of type I interferon (IFN-α/β) protein, resulting in IFN-induced inhibition of FMDV replication. Here we show that in addition to preventing IFN-α/β protein synthesis, L^{pro} reduces the level of immediate-early induction of IFN-β mRNA and IFN-stimulated gene products such as double-stranded RNA-dependent protein kinase R (PKR), 2′,5′-oligoadenylate synthetase, and Mx1 mRNAs in swine cells. Down-regulation of cellular PKR by RNA interference did not affect wild-type virus yield but resulted in a higher yield of A12-LLV2, indicating a direct role of PKR in controlling FMDV replication in the natural host. The observation that L^{pro} controls the transcription of genes involved in innate immunity reveals a novel role of this protein in antagonizing the cellular response to viral infection.

Foot-and-mouth disease (FMD) is a highly contagious viral disease of wild and domestic cloven-hoofed animals, including swine and cattle, that is characterized by temporary and debilitating oral and pedal vesicles. Countries where the disease is enzootic can suffer severe economic losses as a result of a decline in livestock production and international restrictions on exports of animals and animal products, making FMD the most economically important disease of livestock worldwide (17).

The causative agent, FMD virus (FMDV), belongs to the *Aphthovirus* genus of the *Picornaviridae* family and contains a single-stranded, positive-sense RNA genome of approximately 8,500 nucleotides surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins (VP1 [1D], VP2 [1B], VP3 [1C], and VP4 [1A]) (17, 42, 43). Upon infection, the viral RNA is translated as a single, long open reading frame into a polyprotein that is cotranslationally processed by three virus-encoded proteinases, leader (L^{pro}), 2A, and $3C^{pro}$, into the four structural proteins and a number of nonstructural proteins, which function in various aspects of the replication cycle (31, 43). L^{pro}, the first viral protein translated, is a papain-like proteinase (24, 36, 41, 46) that cleaves itself from the polyprotein precursor and also cleaves host translation initiation factor eIF-4G, resulting in the shut-off of host cap-dependent mRNA translation (13, 23, 32, 49). FMDV mRNA, in contrast, is translated by a cap-independent mechanism via an internal ribosome entry site and does not require intact eIF-4G for viral protein production (2, 26). Thus, as a result of FMDV infection, host cell protein synthesis is rapidly shut off without affecting translation of viral mRNA, thereby diverting the cell protein synthesis machinery to the production of large amounts of virus.

To examine the potential role of L^{pro} in pathogenesis, we constructed a virus lacking this coding region (leaderless virus A12-LLV2, a genetically engineered FMDV lacking the L^{pro} coding region) (36). Surprisingly leaderless virus grew almost as well as wild-type (WT) virus in some cell lines including BHK-21 and swine IBRS-2 cells, suggesting that L^{pro} is not required for growth in cell culture. However, in contrast to WT virus, leaderless virus is highly attenuated in both cattle and swine (4, 30), and after aerosol infection of cattle, it does not spread systemically beyond the initial site of infection in the lungs (4). Based on this information, we proposed that L^{pro} is an important virulence factor in livestock hosts.

To understand the molecular basis for the difference in virulence of leaderless virus between cell culture and susceptible animals, we screened a number of secondary cells for their ability to differentially support the growth of WT and leaderless virus. We identified swine, bovine, and lamb cells in which leaderless virus infection does not result in plaque formation, causes only limited cytopathic effect (CPE), and produces significantly lower virus yields than WT virus infection (6, 7), correlating with the inability of leaderless virus to spread systemically in the animal. We found that these cells have an active type I interferon (IFN-α/β) system, while BHK-21 and IBRS-2 cells do not (6, 7). Supernatants from leaderless virus-infected secondary cells contained higher levels of antiviral activity than supernatants from WT virus-infected cells, and this activity is IFN-α/β specific (6). Utilizing embryonic fibroblasts derived from knockout mice, we showed that two IFN-α/β-stimulated gene (ISG) products, double-stranded RNA-

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dependent protein kinase R (PKR) and RNase L, are involved in the inhibition of FMDV replication (7). These results suggested that in WT virus-infected secondary cells and in susceptible animals, L^{pro} inhibits the translation of capped host mRNAs, including IFN-α/β mRNAs, thereby blocking or reducing the innate immune response to virus infection (3, 7). As a result, FMDV rapidly replicates and spreads. In contrast, in leaderless virus-infected cells, the absence of L^{pro} allows the translation of IFN-α/β mRNA and IFN protein secretion. Binding of IFN protein to its receptor induces an antiviral state through paracrine and autocrine processes that lead to activation of ISG products, some of which, including PKR and RNase L, inhibit FMDV replication (3, 7, 44, 45).

Among the *Picornaviridae* family only cardioviruses, including mengo and Theiler's viruses, also encode an L protein (43). The L protein of these viruses does not have any known proteinase activity, but this protein has been reported to be an essential factor in virus replication in IFN-α/β-competent cells (25, 55) and is required for neurovirulence of the GDVII strain of Theiler's virus (5). More recently, cardiovirus L protein has been associated with the inhibition of IFN-α/β production (11, 50, 55, 56). Thus, similar to FMDV L^{pro}, cardiovirus L also appears to have an important role in pathogenesis.

To more closely examine the effect of WT and leaderless virus infection on the induction of the host IFN-α/β response, we have followed the synthesis of IFN-α/β mRNA and three well-characterized ISG products, PKR, 2′,5′-oligoadenylate synthetase (OAS), and Mx1 (3, 44, 45), in swine cells with an active IFN system. Infection with leaderless virus resulted in significantly higher levels of IFN-β mRNA, suggesting a direct effect of WT virus on IFN-β mRNA transcription. Augmented levels of IFN-β correlated with enhanced induction of PKR, OAS, and Mx1 mRNAs and increased levels of antiviral activity in the supernatant of leaderless virus-infected cells. Utilizing RNA interference to knock down PKR mRNA expression, we confirmed the role of this gene product as an inhibitor of FMDV replication in swine cells. These results show that L^{pro} down-regulates the innate immune response to FMDV infection at both the transcriptional and translational levels.

MATERIALS AND METHODS

Cells and viruses. Porcine kidney cell lines, IBRS-2 and SK6, were obtained from the Foreign Animal Disease Diagnostic Laboratory, Plum Island Animal Disease Center, Greenport, N.Y. Secondary porcine kidney (PK) cells were provided by Animal Plant and Health Inspection Service, National Veterinary Service Laboratory, Ames, Iowa. These cells were maintained in minimal essential medium (MEM; Gibco BRL/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and supplemented with 1% antibiotics and nonessential amino acids. BHK-21 cells (baby hamster kidney cells strain 21, clone 13; ATCC CL10), obtained from the American Type Culture Collection (Rockville, MD) were used to propagate virus stocks and to measure virus titers by plaque assay. They were maintained in MEM containing 10% calf serum and 10% tryptose phosphate broth supplemented with 1% antibiotics and nonessential amino acids. All cell cultures were incubated at 37°C in 5% CO_2.

FMDV A12-IC (WT virus) was generated from the full-length serotype A12 infectious clone, $pRMC_{35}$ (40), and A12-LLV2 (leaderless virus) was derived from the infectious clone lacking the Lb coding region, pRM-LLV2 (36). Viruses were concentrated by polyethylene glycol and maintained at −70°C. For all experiments virus titers were measured on BHK-21 cells.

Viral assays. (i) Single-step growth titration. SK6 and BHK-21 cells were infected with FMDV WT and A12-LLV2 at a multiplicity of infection (MOI) of 10 at 37°C. After a 1-h adsorption step, cells were rinsed twice with 150 mM NaCl, 20 mM morpholineethanesulfonic acid (MES; pH 6) to inactivate unadsorbed input virus and twice with MEM to neutralize the MES, followed by addition of MEM and incubation at 37°C. Supernatants were collected at 1 and 24 h postinfection (hpi) and titrated in BHK-21 cells (6).

(ii) FMDV infections. Porcine cell monolayers in six-well plates were infected, in duplicate, with FMDV WT and A12-LLV2 at different MOIs (1 or 10) for 1 h at 37°C. After adsorption, cells were rinsed and incubated with MEM at 37°C. Supernatants and cell lysates were collected at different times postinfection (0, 1, 3, 6, 11, and 24 hpi) for further analysis. A control with mock-infected cells was prepared at each time point. When indicated, cycloheximide was added to the medium and maintained throughout the course of the experiment.

IFN assays. (i) Biological activity. Supernatants from SK6 or PK cells infected with WT or A12-LLV2 were centrifuged to remove cell debris, treated at pH 2 overnight on ice to inactivate any residual FMDV, and neutralized to pH 7 (6). IBRS-2 cells were incubated for 20 to 24 h with the above supernatants and subsequently infected with WT FMDV. The virus was adsorbed for 1 h at 37°C, and the cells were overlaid with gum tragacanth and incubated for 24 h at 37°C. Plaques were visualized by staining with crystal violet (6). Antiviral activity of IFN was reported as the reciprocal of the highest sample dilution that resulted in a 50% reduction in the number of plaques relative to the number of plaques of the untreated cells.

(ii) Porcine IFN-α ELISA. A porcine IFN-α double-capture enzyme-linked immunosorbent assay (ELISA) previously developed in our laboratory was used to quantitate IFN-α protein in the supernatants of infected cells (34). Porcine IFN-α monoclonal antibodies (MAb) K9 and F17 were purchased from R&D Systems (Minneapolis, MN). MAb K9 (1 μg/ml) was used for antigen capture and biotinylated MAb F17 (0.35 μg/ml) in conjunction with horseradish peroxidase-conjugated streptavidin (KPL, Gaithersburg, MD) were used for detection. pIFN-α concentrations were determined by extrapolation on a standard curve prepared with recombinant pIFN-α (PBL Biomedical Laboratories, Piscataway, NJ).

(iii) Porcine IFN-β ELISA. The levels of IFN-β protein in the supernatants of FMDV-infected cells were measured by an antigen capture ELISA. Rabbit polyclonal antipeptide antibodies to the N- and C-termini of porcine IFN-β were prepared by Zymed Laboratories (Invitrogen, Carlsbad, CA). Anti-N-pIFN-β (2 μg/ml) was used for antigen capture, and biotinylated anti-C-pIFN-β (1 μg/ml) in conjunction with streptavidin-horseradish peroxidase (KPL) was used for detection. The amounts of pIFN-β were indicated in arbitrary units calculated by extrapolation on a standard curve prepared with pIFN-β expressed from a recombinant replication-defective human adenovirus type 5 vector in IBRS-2 cells (8). The biological activity of this recombinant protein was determined as described above (see "Biological activity").

Analysis of IFN and ISG mRNA. A quantitative real-time reverse transcription-PCR (RT-PCR) assay was used to evaluate the mRNA levels of the porcine IFN-α, IFN-β, PKR, OAS, and Mx1 genes. RNA was extracted from monolayers of porcine cells [FMDV-infected, mock-infected, or treated with 10 μg/ml poly(rI · C) in the presence of Lipofectamine 2000 (Invitrogen)] collected at different times postinfection by using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Approximately 1 μg of RNA was treated with DNase I (Sigma, St Louis, MO) and used to synthesize cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers following the manufacturer's directions. An aliquot (1/40) of the cDNA was used as a template for real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and TaqMan minor groove binding probes were designed with Primer Express software, version 1.5 (Applied Biosystems). Forward and reverse primers were purchased from Invitrogen and the FAM (6-carboxyfluorescein)-labeled TaqMan minor groove binding probes were from Applied Biosystems. 18S rRNA (Applied Biosystems) or porcine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the values for each sample. The sequences for the primers and probes are listed in Table 1. Reactions were performed in an ABI Prism 7700 Sequence detection system (Applied Biosystems).

RNA interference (RNAi) for porcine PKR. Specific small interfering RNAs (siRNAs) were designed and synthesized by Dharmacon (Chicago, IL) using the sequence of porcine PKR (NCBI GenBank accession code NM_214319). For all experiments, a pool containing four siRNAs targeting the PKR coding sequence (5′-GCACATAACTTGAGGTTTA-3′, 5′-GGAAGAACGTCACAGAGAA-3′, 5′-GAGCAAAGCATGAATACTT-3′, and 5′-CCTGAAGGCTGGCGTCTTA-3′) was used. siGLO (Control Risc-free; Dharmacon) was included as a negative control. Twelve-well plates were seeded with 10^5 SK6 cells/well in MEM containing 10% FBS and supplements. When the cells were about 80 to 90% confluent, the medium was replaced by OptiMEM I (Invitrogen), and after 4 h the pool of siRNAs (200 nM) was transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. FBS (0.5%) was added 4 h

TABLE 1. Oligonucleotide primer and probe sequences for amplification of porcine genes used in real-time RT-PCR

Gene type	Primer and probe sets	Sequence	GenBank[a]
GAPDH	Porcine GAPDH-327F[b]	5′-CGTCCCTGAGACACGATGGT-3′	AF017079
	Porcine GAPDH-380R[c]	5′-CCCGATGCGGCCAAAT-3′	
	Porcine GAPDH-348T[d]	5′-AAGGTCGGAGTGAACG-3′	
Mx1	Porcine Mx1-803F[b]	5′-GAGGTGGACCCCGAAGGA-3′	M65087
	Porcine Mx1-859R[c]	5′-CACCAGATCCGGCTTCGT-3′	
	Porcine Mx1-824T[d]	5′-AGGACCATCGGGATC-3′	
OAS	Porcine OAS-889F[b]	5′-CTGTCGTTGGACGATGTATGCT-3′	AJ225090
	Porcine OAS-954R[c]	5′-CAGCCGGGTCCAGAATCA-3′	
	Porcine OAS-919T[d]	5′-TCAAGAAACCCAGGCCT-3′	
PKR	Porcine PKR-968F[b]	5′-GGAAGAAAACAAACACAGCTTGAA-3′	AB104654
	Porcine PKR-1048R[c]	5′-CCAAATCCACCTGAGCCAATT-3′	
	Porcine PKR-994T[d]	5′-CCAGGTT TGTCGAAGAT-3′	
pIFNα set#1	Porcine IFNα-236F[b]	5′-TGGTGCATGAGATGCTCCA-3′	M28623
	Porcine IFNα-290R[c]	5′-GCCGAGCCCTCTGTGCT-3′	
	Porcine IFNα-256T[d]	5′-CAGACCTTCCAGCTCT-3′	
pIFNα set#2	Porcine IFNα-449F[b]	5′-TCACCCTCTATCTGCAAGAGAAGA-3′	M28623
	Porcine IFNα-514R[c]	5′-TGACTTCTGCCCTGACGATCT-3′	
	Porcine IFNα-478T[d]	5′-AGCCCTGTGCCTG-3′	
pIFNβ	Porcine IFNβ-11F[b]	5′-AGTGCATCCTCCAAATCGCT-3′	M86762
	Porcine IFNβ-69R[c]	5′-GCTCATGGAAAGAGCTGTGGT-3′	
	Porcine IFNβ-32T[d]	5′-TCCTGATGTGTTTCTC-3′	

[a] NCBI GenBank accession code.
[b] Forward primer.
[c] Reverse primer.
[d] TaqMan FAM-MGB probe.

posttransfection. A second transfection under identical conditions was performed after 24 h, and transfected cells were subsequently infected with FMDV.

RESULTS

Replication of WT and A12-LLV2 in porcine kidney cells. We previously reported that secondary cells with a competent type I IFN system (embryonic bovine kidney, lamb kidney, and PK cells) differentially support the growth of WT versus leaderless FMDV (6). To facilitate further experiments, we tested an established swine cell line, SK6, for its susceptibility to FMDV and its ability to inhibit leaderless virus spread. In parallel, we used BHK-21 and secondary PK cells as controls. When SK6 cells were infected with WT virus at an MOI of 10, we observed a CPE beginning at 4 to 6 hpi, and by 24 hpi most of the monolayer was affected. For A12-LLV2-infected SK6 cells, CPE started at approximately 9 to 10 hpi, and by 24 hpi 80 to 90% of the cells were still unaffected. Infection of secondary PK cells produced similar results, but the effects were more pronounced. In addition, no plaques were visualized in A12-LLV2-infected SK6 or PK cells in contrast to WT virus-infected cells (data not shown). In BHK-21 cells, which have an impaired IFN system, A12-LLV2 grew and formed plaques almost as efficiently as WT virus.

A single-step growth experiment was performed in SK6 cells to determine if A12-LLV2 could replicate despite its inability to form plaques in this cell line. BHK-21 cells were used as a control. Both cell lines were infected with WT and A12-LLV2 viruses, and supernatants were collected for titration on BHK-21 cells. A12-LLV2 grew to lower titers than WT virus in both cell lines (Fig. 1). However, the yield of A12-LLV2 was approximately 50-fold lower than WT in SK6 cells but only 5-fold lower in BHK-21 cells. These results suggested that, similar to our previous studies with secondary cells, the SK6 cell line differentially supports the growth of WT and leaderless virus, making this cell line suitable for further studies.

Antiviral response in host cells. To evaluate the factors involved in the reduced ability of A12-LLV2 to grow in porcine cells, we analyzed supernatants from infected SK6 and PK cells for antiviral activity. Only the supernatants from the A12-LLV2-infected porcine cells contained detectable antiviral activity (Table 2). The maximum inhibitory effect was 16 U with supernatants from A12-LLV2-infected SK6 cells (MOI of 1; 30 hpi) and A12-LLV2-infected PK cells (MOI of 1; 24 hpi). Mock- or WT virus-infected cells produced less than 2 U of biological activity by 30 hpi. To confirm that the antiviral activity was IFN-α/β specific, supernatants from infected SK6 cells were pretreated with anti-IFN-α, anti-IFN-β, and a combination of anti-IFN-α and anti-IFN-β antibodies or with nor-

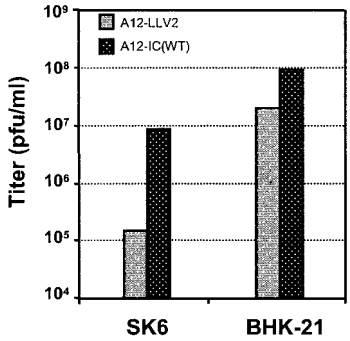

FIG. 1. End-point titration. BHK-21 and SK6 cells were infected with A12-IC (WT) or A12-LLV2 at an MOI of 10 at 37°C. After 1 h, unabsorbed virus was removed by washing with 150 mM NaCl–20 mM MES (pH 6.0), and MEM was added. Supernatants were collected at 24 hpi, and the virus titers were determined on BHK-21 cells.

TABLE 2. Antiviral activity in supernatants of porcine cells[a]

Inducing virus[b]	Total antiviral activity (U)	Antiviral activity (U) in cells incubated with:			
		Normal serum	Neutralizing anti-IFN-α	Neutralizing anti-IFN-β	Neutralizing anti-IFN-α/β
A12-LLV2	16	16	<2	16	<2
A12-IC (WT)	<2	<2	NA	NA	NA

[a] Supernatants from infected SK6 cells were incubated with serum for 1 h at RT prior to determination of antiviral activity. Activity is measured in arbitrary units. NA, not applicable.
[b] Virus was used at an MOI of 1.

mal serum as a control. We had previously demonstrated that these antibodies neutralize the biological activity of their respective antigens (data not shown). All the antiviral activity was neutralized with anti-IFN-α or anti-IFN-α plus anti-IFN-β antibodies. Anti-IFN-β alone or normal serum did not result in any decrease of the antiviral activity, indicating that only IFN-α-derived activity was present in the supernatants of infected cells.

We measured the levels of IFN-α/β protein in supernatants of porcine cells by an antigen capture ELISA (Fig. 2). IFN-α accumulated to higher levels in the supernatants of both PK and SK6 cells infected with leaderless virus compared to WT virus. The level of IFN-α was approximately 10-fold higher in A12-LLV2-infected SK6 cells than in WT virus-infected cells at 30 hpi (Fig. 2A) and about 5-fold higher in leaderless virus-infected PK cells than in WT virus-infected cells at 24 hpi (Fig. 2B). IFN-β protein was not detected in the supernatants from any tested cell type (data not shown).

PKR is required to achieve an antiviral state in porcine cells. Using embryonic fibroblasts derived from WT and knockout mice, we have previously shown that resistance to FMDV infection is partially dependent upon the presence of PKR (7). To confirm the role of this gene in the antiviral response observed in porcine cells, we used RNAi to reduce the levels of PKR mRNA. We used a pool of four specific siRNAs that reduced the levels of PKR mRNA by approximately 80% as measured by real-time RT-PCR (data not shown). SK6 cells transfected with PKR-specific siRNAs were infected with WT or A12-LLV2 at different MOIs. As controls we included non-transfected cells or cells transfected with a nonspecific siRNA (siGLO). When control siRNA (siGLO) was transfected, the yield of leaderless virus was between 1.1×10^3 and 3.9×10^4 PFU/ml compared to 1.1 and 2.3×10^7 PFU/ml for the WT virus, a relative difference of approximately 600- to 10,000-fold depending on the MOI of challenge (Fig. 3A). However, in the presence of specific siRNA (siPKR), the yield of A12-LLV2 increased significantly, approximately 8- to 120-fold, whereas the yield of WT virus was essentially unaffected (Fig. 3B). These results indicate that, similar to mouse cells, PKR is required for the antiviral activity of porcine cells against FMDV.

Induction of type I IFN mRNA following WT and A12-LLV2 infection of porcine cells. As we previously mentioned, the attenuated phenotype of A12-LLV2 in cells with a competent IFN system is due to the inability of this virus to block host cell translation; hence, there is an increase of secreted IFN protein and antiviral activity. To study whether the expression of IFN-α/β mRNA in infected porcine cells was also differentially affected by these viruses, we measured the levels of the corresponding mRNAs by real-time RT-PCR. A12-LLV2 induced approximately 10- to 30-fold higher levels of IFN-β mRNA at 6 to 11 hpi in both SK6 and PK cells compared to WT virus (Fig. 4A and B). No significant changes in the levels of IFN-α1 mRNA were detected early after infection with FMDV (Fig. 4C and D). However, at late times, 24 to 30 hpi, we observed an increase of IFN-α1 mRNA only in WT virus-infected cells (six- to eightfold). In most species IFN-α constitutes a family of closely related genes. However, for porcine genes the only sequence available is IFN-α1. To verify our results, we repeated the real-time PCR assay by using an alternative set of specific primers (Table 1, IFN-α1 set 2). Consistent with the real-time PCR results with primer set 1, IFN-α1 mRNA induction was only detected in WT virus-infected cells at late times after infection (data not shown). Amplification of a larger region of the IFN-α1 gene (primers pIFN-α 236F and pIFN-α 514R) by semiquantitative RT-PCR showed a band of the expected size for WT- and A12-LLV2-infected cells at early and late times postinfection (data not shown). No induction of IFN-β/α1 mRNA was observed upon infection with UV-inactivated WT or leaderless virus, suggesting that viral replication was required for this function (data not shown).

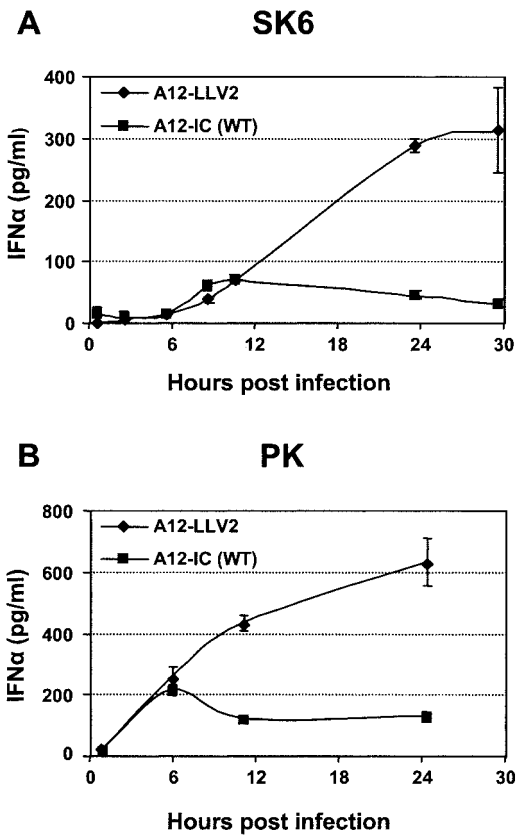

FIG. 2. IFN secretion after FMDV infection. SK6 (A) or PK (B) cells were infected with A12-IC (WT) or A12-LLV2 at an MOI of 1. Supernatants were collected at different times postinfection, and pIFN-α was measured by antigen capture ELISA. The results are representative of three independent experiments. Error bars refer to the standard deviation for each mean data point ($n = 3$).

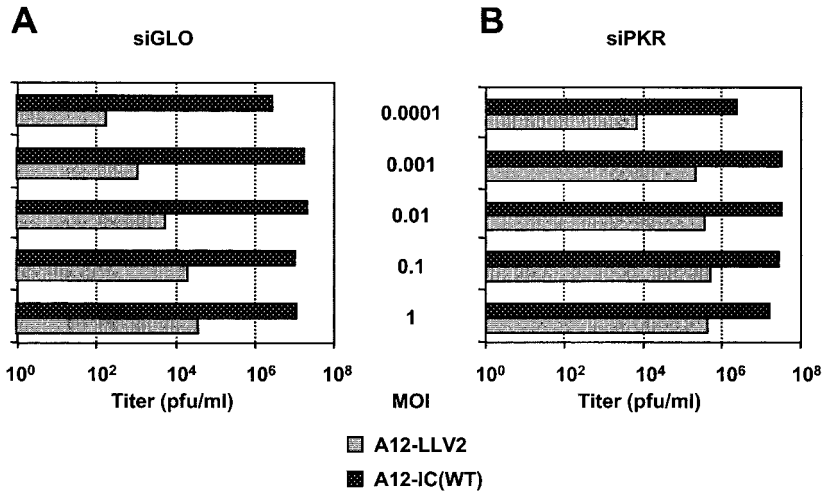

FIG. 3. RNAi of porcine PKR. SK6 cells were transfected twice with 200 nM of a pool of siRNAs specific for porcine PKR (siPKR). Transfected cells were infected with A12-IC (WT) or A12-LLV2 at MOIs from 0.0001 to 1, and after 24 h the virus titer was measured on BHK-21 cells (B). A control experiment with siGLO (Risc-free) was performed simultaneously (A). The results are representative of two independent experiments.

Induction of IFN-β expression is independent of de novo protein synthesis (22, 38, 39). However, since WT FMDV blocks host cell translation, it might be argued that the decreased induction of IFN-β mRNA expression in WT virus-infected cells compared to leaderless virus-infected cells is simply due to the inhibition by L^{pro} of the synthesis of a protein factor that is required for IFN-β mRNA transcription. To confirm that induction of IFN-β mRNA transcription takes place in porcine cells despite the inhibition of protein synthesis and that WT FMDV blocks this activation more efficiently than leaderless virus, we measured the levels of mRNA induction in the presence of cycloheximide. We performed parallel experiments using poly(rI · C) or virus as inducers. Poly(rI · C) has been shown to be a potent IFN-β transcriptional inducer in multiple cell types (28). As seen in Fig. 5A, the addition of cycloheximide to the culture medium did not block the induction of

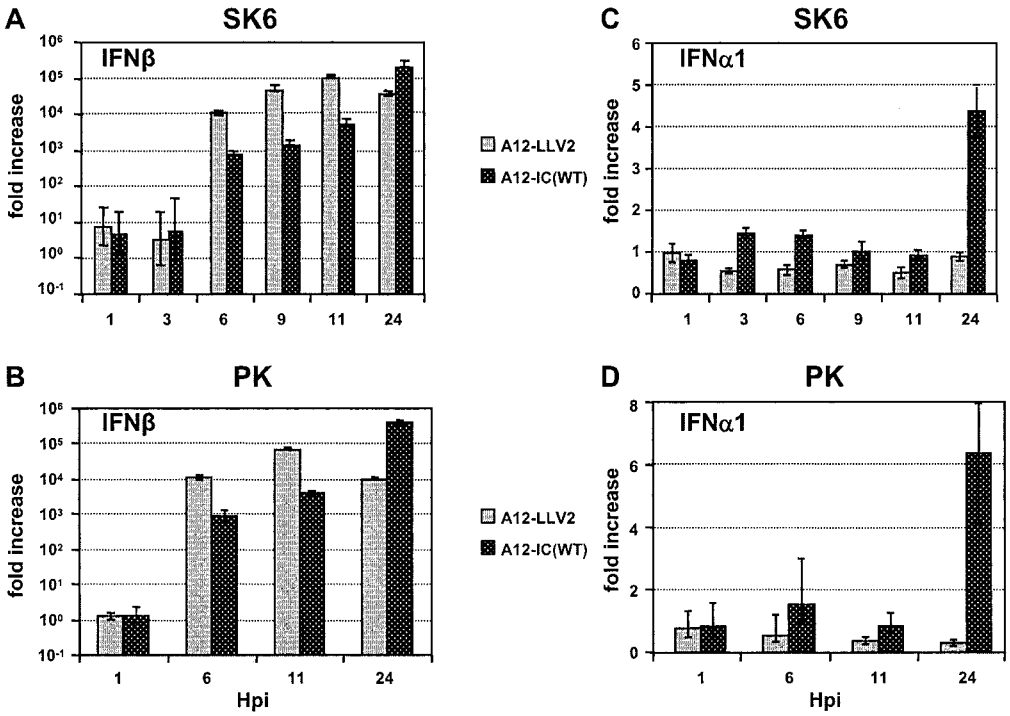

FIG. 4. IFN-α/β response in porcine cells. IFN-α1/β mRNA expression was measured by real-time RT-PCR in SK6 cells (A and C) or PK cells (B and D) infected at an MOI of 1 with A12-IC (WT) or A12-LLV2. Porcine GAPDH was used as an internal control. Results are expressed as the increase (*n*-fold) of gene expression for virus-infected cells relative to mock-infected cells at the indicated time points. Error bars indicate the standard deviation for each mean data point ($n = 3$). Similar results were obtained in at least two independent experiments.

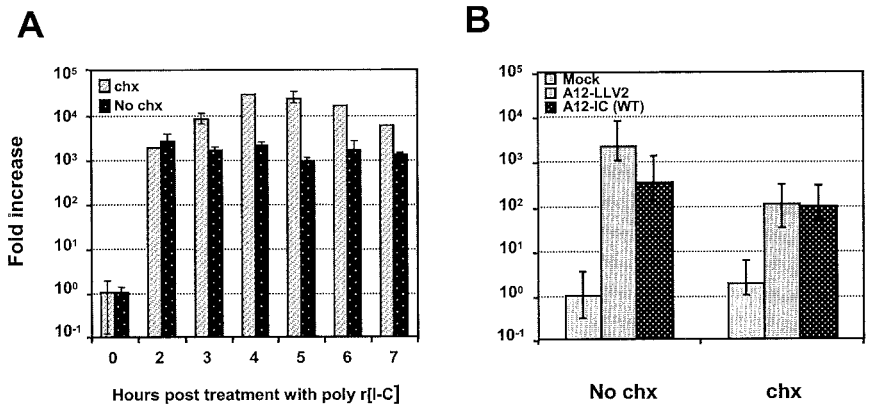

FIG. 5. Induction of IFN-β mRNA expression in the presence of cycloheximide. (A) SK6 cells were treated with 10 μg/ml of poly(rI · C) in the absence or presence of 100 μg/ml cycloheximide. (B) SK6 cells were infected with A12-IC (WT) or A12-LLV2 (MOI of 1; 9 h) in the absence or presence of 50 μg/ml cycloheximide. The levels of IFN-β mRNA were measured by real-time RT-PCR as indicated in Fig. 4. Error bars indicate the standard deviation for each mean data point (n = 3). chx, cycloheximide.

IFN-β mRNA transcription in poly(rI · C)-stimulated cells; instead there was an increase in the level of induction in SK6 cells even at 7 h after the drug was added. These results suggest that factors required for IFN-β mRNA transcription are constitutively expressed or that cycloheximide blocked the expression of a possible repressor. Similar results have been previously reported and termed superinduction (37). As shown in Fig. 4, in the absence of cycloheximide, the leaderless virus was a better inducer of IFN-β mRNA than WT virus in SK6 cells, by approximately eightfold (Fig. 5B). However, in the presence of cycloheximide, the induction of IFN-β mRNA was the same for both viruses, but the overall response was lower, consistent with the inhibition of viral replication and viral double-stranded RNA production. These results suggest that in infected cells cycloheximide inhibits the expression of a factor(s) required for WT virus to block the induction of IFN-β mRNA.

Expression of ISGs following WT and A12-LLV2 infection of porcine cells. IFN-α/β is able to induce more than 300 ISGs with antiviral and immunomodulatory functions (12, 14). Among them PKR, OAS, and Mx1 have been studied in detail and are induced by IFN after virus infection (19, 48, 52, 54). We analyzed the synthesis of mRNA for these three genes by real-time RT-PCR after infection of SK6 and PK cells with WT and A12-LLV2. As seen in Fig. 6, both viruses increased the expression of all three mRNAs in SK6 cells. However, the leaderless virus was a better inducer than WT virus: approximately 4-fold higher for PKR, 18- to 43-fold higher for OAS, and 10- to 20-fold higher for Mx1. A similar pattern was observed in infected PK cells. Induction of all three genes was delayed by approximately 2 to 3 h with respect to the induction of IFN-β, suggesting that these cells responded to increased levels of IFN protein acting in an autocrine or paracrine manner.

DISCUSSION

In this report we show that in addition to inhibiting host protein synthesis, FMDV is capable of interfering with the immediate-early virus-induced transcription of IFN-β mRNA. Infection with leaderless virus, which lacks the L^{pro} coding region, induced higher levels of IFN-β mRNA than infection with WT virus, suggesting a direct role of the leader protein in this inhibition. This conclusion was further supported by the observation that the addition of cycloheximide during infection by leaderless or WT virus resulted in reduced but almost identical amounts of IFN-β mRNA.

Most viruses have developed mechanisms to escape the IFN system (1, 9, 15, 16, 20, 51). They can interfere with IFN expression, inhibit IFN-activated cellular signaling, or perturb the action of IFN-induced antiviral factors. The L protein of Theiler's virus inhibits the expression of IFN-α4 and IFN-β mRNAs (50) by blocking the translocation of IFN regulatory factor 3 to the nucleus (11). In addition the L protein of mengo virus blocks the induction of IFN-α/β by preventing the activation of NF-κB (56).

FMDV infection selectively induced IFN-β mRNA, since no early induction of IFN-α1 was observed. The levels of IFN-α1 mRNA increased at late time postinfection (24 h), predominantly in WT virus-infected cells. It is noteworthy to mention that the primers used were selected from the sequences of the single porcine IFN-β and IFN-α1 genes currently available (Table 1). In several species, IFN-β is encoded by a single gene. In contrast, IFN-α constitutes a family of structurally related genes (18, 21). Within these IFN-α families, murine IFN-α4 and human IFN-α1 play a unique role in the innate immune response against viruses, since they are induced early during viral infection without the requirement of protein synthesis, whereas other IFN-α subtypes require newly synthesized protein (29). For porcine IFN-α there are at least 10 potential genes or pseudogenes, but only IFN-α1, IFN-ω, and two IFN-α-pseudogenes have been cloned and completely sequenced (27, 33). Our results indicate that in secondary porcine cells or in SK6 cells, porcine IFN-α1 does not behave as murine IFN-α4 or human IFN-α1 since there is no immediate-early induction after FMDV infection or poly(rI · C) treatment. However, the increase in IFN-α1 expression at late times postinfection suggests that this gene is an active member of the porcine IFN-α gene family. Characterization of all members of this family may help to detect if specific patterns of expression of each IFN-α subtype occur during viral infection.

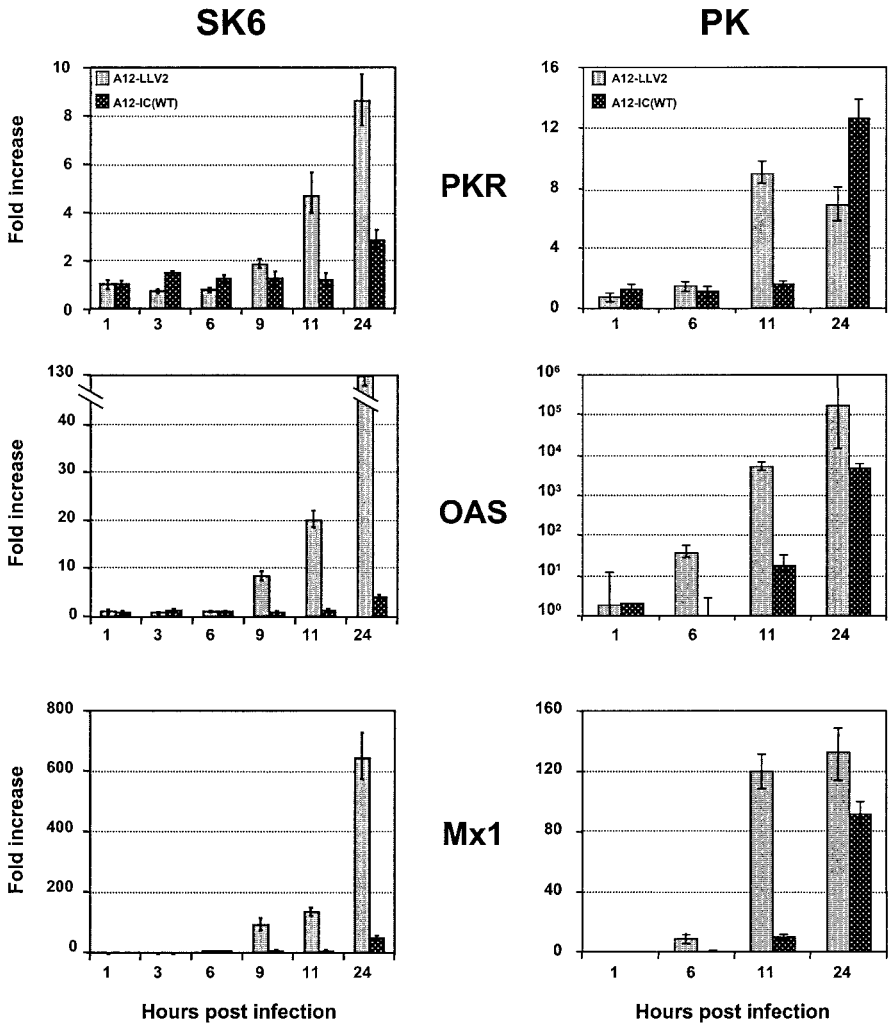

FIG. 6. Expression of ISG products in response to FMDV infection. SK6 or PK cells were infected with A12-IC (WT) and A12-LLV2 at an MOI of 1. Expression of PKR, OAS, and Mx1 mRNAs was measured by real-time RT-PCR using GAPDH or 18S rRNA as an internal control. Results are expressed as the increase (*n*-fold) for each specific gene in virus-infected cells relative to mock-infected cells. Error bars indicate the standard deviation for each mean data point ($n = 3$). Similar results were obtained in at least two independent experiments.

Consistent with the early higher levels of IFN mRNA, A12-LLV2 induced higher levels of antiviral activity in the supernatants of infected cells than WT virus. This activity was evaluated by a biological assay and correlated with the amount of IFN protein present. Only IFN-α protein was detected by ELISA, and this result was confirmed by neutralization of the antiviral activity with a specific anti-IFN-α antibody. Although we could not detect specific induction of IFN-α1 mRNA expression by real-time RT-PCR, semiquantitative RT-PCR analysis of the IFN-α coding sequence allowed us to detect the presence of total IFN-α mRNA during infection (data not shown). However, this assay did not allow us to discriminate among the different members of the porcine IFN-α family, nor did it show a differential response between WT and leaderless viruses. Thus, the higher level of IFN-α protein in leaderless virus-infected cells compared to WT virus-infected cells correlates with the inability of A12-LLV2 to block host cell translation.

We were unable to detect IFN-β protein in the supernatants of leaderless or WT virus-infected cells by an antiviral assay (Table 2), ELISA, Western blotting, or immunoprecipitation of radiolabeled infected cell lysates or supernatants (data not shown). Previous reports have indicated that there are viral factors that can influence the translatability of IFN-β mRNA in Sendai virus-infected cells (10). Furthermore, other studies have suggested that the stability of IFN-β mRNA could be posttranscriptionally modified by deadenylation (35). In addition, studies with human respiratory syncytial virus have shown that only low levels of secreted IFN-β protein were detectable in the supernatants of infected human epithelial cells or macrophages, despite the high levels of IFN-β mRNA present in these cells (47). Perhaps in some cell types only a very small proportion of the IFN-β mRNA is translated or the turnover rate of IFN-β mRNA/protein is rapid.

We followed the kinetics of expression of three well-characterized ISG products, PKR, OAS, and Mx1, in WT- and A12-LLV2 virus-infected porcine cells. As expected, induction of transcription of all three ISGs correlated with induction of transcription of IFNs, but with a delay of approximately 2 to

3 h, a time period that may be required for the expression and secretion of IFN protein. A12-LLV2 was a more potent inducer of these ISGs than WT virus. This effect is in agreement with the hypothesis that L^{pro} blocks the induction of transcription and translation of IFN mRNA, resulting in a decrease in the expression of ISGs compared to LLV2. However, the overall response, even in the presence of L^{pro}, was the up-regulation of the ISG mRNAs. Nevertheless, despite the induction of antiviral pathways, the block in host cell translation apparently prevents an efficient antiviral response to WT virus infection.

The role of PKR in the IFN-induced inhibition of A12-LLV2 replication was confirmed in porcine cells. RNAi of PKR in SK6 cells resulted in a significant growth advantage for A12-LLV2. The yield of leaderless virus increased approximately 8- to 120-fold in cells with reduced expression of PKR in contrast to the yield of WT virus, which was essentially unaffected.

FMDV infection blocks the antiviral activity of IFN-α/β by limiting the transcription of IFN-β and inhibiting the translation of IFN-α/β mRNA. Currently, we do not know whether the proteinase activity of leader is directly involved in inhibition of transcription or if L^{pro} utilizes an alternative mechanism to affect some stage in the activation of one or more transcription factors required for IFN-β expression. Nevertheless, FMDV replication is inhibited by pretreatment of cells with IFN-α/β protein, suggesting that this virus cannot effectively counteract the antiviral activity of expressed ISG products (7). Indeed, we have recently shown that pretreatment of swine with IFN-α can sterilely protect these animals from direct virus challenge (8, 34) but only delays and reduces the severity of disease in cattle (53). Thus, understanding in detail the interaction of this virus with the complex network of the innate immune system could allow us to rationally develop a more effective disease control strategy for all susceptible animals.

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