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Classical Swine Fever Virus Interferes with Cellular Antiviral Defense: Evidence for a Novel Function of N^{pro}

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Classical swine fever virus (CSFV) replicates efficiently in cell lines and monocytic cells, including macrophages (MΦ), without causing a cytopathic effect or inducing interferon (IFN) secretion. In the present study, the capacity of CSFV to interfere with cellular antiviral activity was investigated. When the porcine kidney cell line SK-6 was infected with CSFV, there was a 100-fold increased capacity to resist to apoptosis induced by polyinosinic-polycytidylic acid [poly(IC)], a synthetic double-stranded RNA. In MΦ, the virus infection inhibited poly(IC)-induced alpha/beta IFN (type I IFN) synthesis. This interference with cellular antiviral defense correlated with the presence of the viral N^{pro} gene. Mutants lacking the N^{pro} gene (ΔN^{pro} CSFV) did not protect SK-6 cells from poly(IC)-induced apoptosis, despite growth properties and protein expression levels similar to those of the wild-type virus. Furthermore, ΔN^{pro} CSFV did not prevent poly(IC)-induced type I IFN production in MΦ but rather induced type I IFN in the absence of poly(IC) in both MΦ and the porcine kidney cell line PK-15, but not in SK-6 cells. With MΦ and PK-15, an impaired replication of the ΔN^{pro} CSFV compared with wild-type virus was noted. In addition, ΔN^{pro} CSFV, but not wild-type CSFV, could interfere with vesicular stomatitis virus replication in PK-15 cells. Taken together, these results provide evidence for a novel function associated with CSFV N^{pro} with respect to the inhibition of the cellular innate immune system.

Classical swine fever (CSF) is a highly contagious disease of pigs caused by the classical swine fever virus (CSFV) and leads to important economic losses worldwide. CSFV, together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), form the genus *Pestivirus* within the family *Flaviviridae*. The two other genera of the family are the genus *Flavivirus* and the genus *Hepacivirus* (55). Pestiviruses carry an RNA genome which possesses a 5′ untranslated region (5′UTR), a single large open reading frame (ORF), and a 3′UTR. The 5′UTR functions as an internal ribosomal entry site for cap-independent translation initiation. The ORF encodes a polyprotein of approximately 3,900 amino acids which is processed into 12 mature proteins by virus-encoded and host cell proteases (for a review, see reference 35). The first protein encoded is the nonstructural protein N^{pro}. It exhibits autoproteolytical activity and cleaves itself off the downstream nucleocapsid protein C (43, 47, 56). Interestingly, it has no corresponding counterpart in flaviviruses and hepatitis C virus and was found to be dispensable for virus replication in cell culture (52). The N^{pro} gene of the moderately virulent strain vA187-1 and of the highly virulent strain vEy-37 has been deleted or replaced with the murine ubiquitin gene, which substitutes for N^{pro} in the generation of the authentic nucleocapsid protein. Such mutants lacking the N^{pro} gene (ΔN^{pro} CSFV) displayed no major alteration of growth characteristics in the porcine kidney cell line SK-6 but were avirulent in pigs (52; D. Mayer, M. A. Hofmann, and J. D. Tratschin, submitted for publication).

Monocytes and macrophages (MΦ) are among the main targets for CSFV infection (29, 53). They are potential factors in the spread of CSFV to different tissues, are major reservoirs for infectious virus, and are involved in CSFV-induced immunomodulation (29). Although CSFV replication in MΦ is sensitive to interferon (IFN), the virus does not induce IFN in these cells (28). Considering that MΦ possess the potential to produce alpha/beta IFN (type I IFN) in response to virus infection (39), it is plausible that CSFV may be capable of counteracting cellular antiviral activity. In this vein, Schweizer and Peterhans (44) recently demonstrated that noncytopathogenic (ncp) BVDV interfered with polyinosinic-polycytidylic acid [poly(IC)]-induced apoptosis in primary bovine turbinate cells and IFN induction in bovine MΦ. As CSFV is also ncp, the present work sought to determine the capacity of CSFV to interfere with apoptosis in the swine kidney cell line SK-6 and with IFN-α/β production in porcine MΦ. The results demonstrated that resistance to poly(IC)-induced cell death and control of IFN induction were dependent on the presence of the N^{pro} gene, indicating a function of N^{pro} in innate immune evasion of CSFV.

MATERIALS AND METHODS

Cells and viruses. The porcine kidney cell lines SK-6 (27) (kindly provided by M. Pensaert, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium) and PK-15 (American Type Culture Collection, Manassas, Va.) were propagated in Earle's minimum essential medium supplemented with 7% horse serum. Monocytes were isolated by culture of peripheral blood mononuclear cells (PBMC) from specific pathogen-free pigs at 4×10^6 cells/ml for 18 h in a mixture of Dulbecco's modified Eagle medium, 10% porcine serum, 2 mM L-glutamine, and 25 mM HEPES, as described earlier (29, 34). Then, the adherent cells, representing monocytic cells partially differentiated to MΦ (6), were detached and reseeded at 0.5×10^6 cells/ml in the above medium for 1 h before infection. The highly virulent CSFV strains Eystrup and Brescia were kindly

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provided by H.-J. Thiel, Justus-Liebig-Universität, Giessen, Germany. CSFV strain Riems, a vaccine virus derived from a lapinized C strain (2), was kindly provided by G. Schirrmeier, Riemser Arzneimittel Gmbh, Insel Riems, Germany. CSFV vA187-1 and vEy-37 were derived from the full-length cDNA clones pA187-1 (42), vRiems, and pEy-37 (D. Mayer, M. A. Hofmann, and J. D. Tratschin, submitted for publication), respectively. The ΔN^{pro} CSFV vA187-ΔN^{pro}-Ubi (formerly named vA187-Ubi), in which the N^{pro} gene was replaced by the murine ubiquitin gene in the vA187-1 backbone, has been previously described (52). CSFV vA187-ΔN^{pro}, an alternative ΔN^{pro} CSFV for which the N^{pro} gene was replaced by the residues methionine and glycine and initially described as being noninfectious (52), was reconstructed and resulted in replication-competent virus rescued from in vitro transcripts with high specific infectivity. CSFV vEy-ΔN^{pro} was constructed in analogy to vA187-ΔN^{pro} by deleting the N^{pro} gene in the pEy-37 cDNA clone. All cDNA-derived viruses were obtained by electroporation of SK-6 cells with the respective in vitro transcripts as described elsewhere (37). For the IFN bioassays, vesicular stomatitis virus (VSV) strain Indiana (American Type Culture Collection) was used at a multiplicity of infection (MOI) of 5 50% tissue culture infectious doses ($TCID_{50}$)/cell.

Assay for cell survival after poly(IC) treatment. SK-6 cells were either mock infected or infected with CSFV for 48 to 72 h and then seeded in duplicate columns in a 96-well plate. For each infected culture, the first column was treated with serial dilutions of poly(IC) (Sigma-Aldrich, Inc.) in complete medium, and the second column was mock treated. Cell survival was then monitored at 24 and 48 h after poly(IC) treatment by crystal violet staining. Briefly, cells were washed once with phosphate-buffered saline (PBS) and then fixed and stained for 30 min with 1% formaldehyde–0.5% crystal violet in 30% ethanol. After extensive washing with water, the stained cells were lysed with isopropanol containing 0.6% sodium dodecyl sulfate and 40 mM HCl. Optical density was measured at 595 nm (OD_{595}). To normalize the values between the different virus-infected cultures, the mean OD_{595} of eight untreated wells was set to 100% cell survival for each culture. The percentage of optical density after poly(IC) treatment was then expressed as percent cell survival.

Immunohistochemistry and FCM. For detection of CSFV-infected cells, the E2-specific monoclonal antibody (MAb) HC/TC26 (20) and the NS3-specific MAb C16 (19) (kindly provided by I. Greiser-Wilke, Hannover Veterinary School, Hannover, Germany) were used for the detection of viral antigen in situ and by flow cytometry (FCM) (48). Briefly, mock- and CSFV-infected cells were fixed and permeabilized with a cell permeabilization kit (Harlan Sera-Lab, Crawley Down, United Kingdom) before labeling with either MAb C16 or HC/TC26 (both at 1 μg/0.5×10^6 cells). Rabbit anti-mouse immunoglobulin G conjugated with phycoerythrin (DAKO A/S, Glostrup, Denmark) was used as secondary reagent. The percentage of infected cells was calculated by subtraction of the background values obtained by C16 staining of mock-infected cells from the values of virus-infected cells. For in situ immunohistochemistry in SK-6 cells, monolayers were dried at 37°C for 30 min and fixed at 80°C for 2 h. PBS–0.01% Tween 20 was used as dilution and wash buffer, and the incubations with primary and secondary antibodies were performed at 37°C for 30 min. Bound primary antibody was labeled with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO A/S) and visualized by adding chromogen solution as described previously (36). Viral antigen detection by FCM was performed as previously described (29).

Analysis of apoptosis by FCM. AnnexinV binding on the surface of apoptotic cells expressing phosphatidylserine and propidium iodide (PI) incorporation by dead cells were analyzed by using standard protocols, as previously described (49). Briefly, cells were detached by trypsin treatment, resuspended in PBS at a concentration of 10^5 cells/ml, and labeled with 2 μg of AnnexinV–fluorescein isothiocyanate/ml in 140 mM NaCl–2.5 mM $CaCl_2$–10 mM HEPES (pH 7.4) for 10 min. After addition of 100 ng of PI/ml (Sigma-Aldrich), the samples were analyzed by FCM. For analysis of the sub-G1 DNA content, the cells were fixed with 75% ethanol at 0°C for 2 min. DNA was then stained with 50 μg of PI/ml and treated with 100 μg of RNase cocktail/ml (Promega) for 30 min at 37°C prior to analysis by FCM.

IFN assays. IFN bioactivity was detected with a bioassay based on the antiviral effect against VSV in the porcine kidney cell line PK-15. A confluent monolayer of the cells, seeded in 96-well plates, was incubated with samples for 18 h before VSV infection with an infectious dose resulting in a complete cytopathic effect (cpe) within 24 h in nontreated controls. The cpe was quantified by crystal violet staining as described above. Serial dilutions of recombinant porcine IFN-α (32) (PBL Biomedical Laboratories, Alexis, Switzerland, and kindly provided by B. Charley, INRA, Jouy-en-Josas, France) were prepared in the respective culture medium to establish standard curves for quantification. For neutralization of IFN-α, 250 μl of sample was incubated for 30 min on ice with 1 μl of rabbit polyclonal antibody against porcine IFN-α (>250 neutralization U/μl as determined by the manufacturer; PBL Biomedical Laboratories).

Type I IFN activity was also assayed using an Mx/CAT reporter gene assay described for the quantification of bovine IFN-α/β and kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom) (14). Briefly, MDBK-t2 cells maintained under blasticidin selection were seeded in six-well plates at a density of 10^6 cells/well. After 24 h, samples diluted 1:5 [or 1:40 if poly(IC) was present in the sample] or porcine IFN-α standard were added to the cells in 1 ml of Earle's minimal essential medium supplemented with 7% heat-inactivated fetal calf serum. The cells were then incubated for 24 h prior to lysis and chloramphenicol acetyltransferase (CAT) quantification using a CAT enzyme-linked immunosorbent assay (Roche Biochemicals, Rotkreuz, Switzerland). Rabbit anti-porcine IFN-α antibody completely blocked activity of recombinant porcine IFN-α in both assays (data not shown), validating their use for quantification of bioactive porcine IFN-α. In some experiments, virus was inactivated by dialysis against 100 mM glycine (pH 2) at 4°C for 24 h followed by an overnight dialysis step against PBS. Alternatively, virus infectivity was neutralized by incubation for 1 h on ice with an excess of MAb HC/TC26 (20). Virus inactivation was monitored on SK-6 cells using immunohistochemistry with MAb HC/TC26.

Kinetics of virus replication. Cells were inoculated for 1 h at an MOI of 1 for SK-6 cells and 5 (based on the titer determined on SK-6 cells) for PK-15 cells and MΦ. The cells were then washed with PBS one and five times, respectively, prior to addition of fresh medium. At various times postinfection (p.i.), replication was stopped by freezing the respective cultures to −70°C, and the cells were lysed by two cycles of freezing and thawing. The culture medium was then clarified by centrifugation at $1,000 \times g$ for 10 min at 4°C and stored in aliquots at −70°C. The titer ($TCID_{50}$ per milliliter) was determined by end-point dilution on SK-6 cells and immunoperoxidase staining using MAb HC/TC26 (see above).

Statistical analyses. The statistical analyses used a two-sample Student's *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

CSFV protects SK-6 cells from poly(IC)-induced apoptosis. The capacity of CSFV to interfere with cell death induced in SK-6 cells by the synthetic double-stranded RNA (dsRNA) poly(IC) was analyzed. Survival of uninfected versus CSFV-infected SK-6 cells following poly(IC) treatment was monitored (Fig. 1). Cells infected with CSFV prior to poly(IC) addition showed an elevated viability compared with uninfected cells (Fig. 1A). With the CSFV-infected cells, around 50% cell survival was observed when the poly(IC) concentration was as high as 20 μg/ml; in contrast, uninfected cells did not survive poly(IC) concentrations in excess of 0.1 μg/ml. This higher resistance to poly(IC)-induced cell death was independent of the virus strain used for infection: the highly virulent vEy-37, the moderately virulent vA187-1, and the avirulent Riems strain conferred similar levels of protection. To confirm that all the cells were infected by CSFV in the cultures used for the cell survival assays, cultures parallel to those employed in Fig. 1A were immunostained for viral glycoprotein E2 expression at the time of poly(IC) addition. As shown in Fig. 1B, almost all of the cells stained for this protein, albeit with variable intensity.

In order to demonstrate that the enhanced cell survival to poly(IC) was indeed due to protection from apoptosis, AnnexinV binding, PI incorporation, and sub-G1 DNA content were analyzed. SK-6 cells, either uninfected or infected with vA187-1, were treated with 100 μg of poly(IC)/ml for 40 h prior to FCM analysis. The results of a representative experiment are shown in Fig. 2. In the uninfected cell culture, a five- to sixfold increase of AnnexinV-positive cells was noted following treatment with poly(IC) (Fig. 2A). When the cells had been previously infected with CSFV, this high concentration of

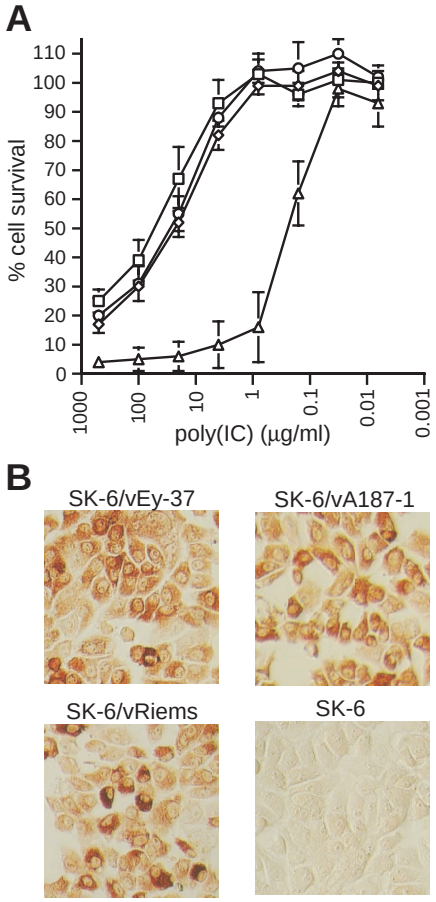

FIG. 1. CSFV-mediated cell survival after poly(IC) treatment is strain independent. Cell survival of SK-6 cells infected with three CSFV strains of different virulence was monitored by crystal violet staining 48 h after poly(IC) treatment. (A) The percentage of cell survival was calculated from the ratio of the OD_{595} of each poly(IC)-treated well to the average OD_{595} of the corresponding eight untreated wells (see Materials and Methods). The mean values with standard deviation from three independent experiments in SK-6 cells infected with CSFV strains vEy-37 (◇), vA187-1 (□), and vRiems (○) or mock-infected cells (△) are shown for decreasing poly(IC) concentrations. (B) Seventy-two hours p.i., at the time of poly(IC) addition, parallel cultures were analyzed for expression of the viral glycoprotein E2 by using immunoperoxidase staining.

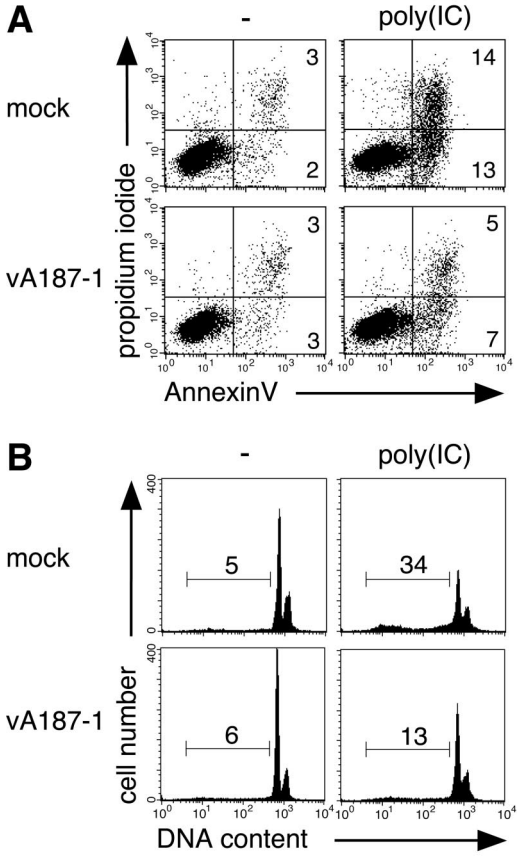

FIG. 2. CSFV protects SK-6 cells from poly(IC)-induced apoptosis. SK-6 cells were infected with CSFV vA187-1 or mock infected and treated after 72 h with 100 μg of poly(IC)/ml or mock treated as control. After 40 h, AnnexinV binding and PI incorporation (A) and sub-G1 DNA content (B) were analyzed by FCM. Numbers in the gated areas indicate the percentage of events. This data are representative of two independent experiments.

poly(IC) induced only a twofold increase in the AnnexinV-positive population (Fig. 2A). Similar ratios were observed for the sub-G1 DNA content (Fig. 2B).

CSFV interferes with poly(IC)-induced type I IFN production in MΦ. For these analyses, MΦ were either mock infected or infected with CSFV strains of different virulence prior to IFN induction by poly(IC). Supernatants from mock- or CSFV-infected MΦ cultures, which did not receive poly(IC), displayed no evidence of antiviral activity either in the VSV assay (Fig. 3) or through detectable CAT expression in the Mx/CAT reporter gene assay (data not shown). The same results were obtained in both assays with and without prior inactivation of the virus present in the MΦ supernatants, demonstrating that wild-type CSFV does not induce antiviral activity or IFN-α/β production in MΦ, PK-15, and MDBK-t2 cells (data not shown). After poly(IC) stimulation of mock-treated MΦ, up to 40 U of type I IFN/ml were detected in eight independent experiments. In contrast, with CSFV-infected MΦ, poly(IC) induced only 0.5 to 3 U of type I IFN/ml. Despite this relatively high variability between the experiments, CSFV-infected MΦ always produced only 2 to 10% of the IFN induced in mock-infected cultures. A representative experiment is shown in Fig. 3. These observations were independent of the CSFV strain used for infection. The type I IFN activity measured in the Mx/CAT reporter gene assay confirmed the results obtained in the VSV assay (data not shown). Furthermore, preincubation of the samples with rabbit anti-porcine IFN-α antibodies abolished the antiviral activity and the CAT response, respectively (data not shown), indicating that the antiviral activity was essentially due to IFN-α. These data demonstrated that both virulent and avirulent CSFV strains were capable of inhibiting the secretion of type I IFN by poly(IC)-treated MΦ.

The replication characteristics of ΔN^{pro} CSFV are dependent on the cell type. It has been previously shown that vA187-ΔN^{pro}-Ubi, a mutant CSFV in which N^{pro} was replaced with the murine ubiquitin gene, replicated to titers similar to those of the parent virus vA187-1 in SK-6 cells (52). Experiments in

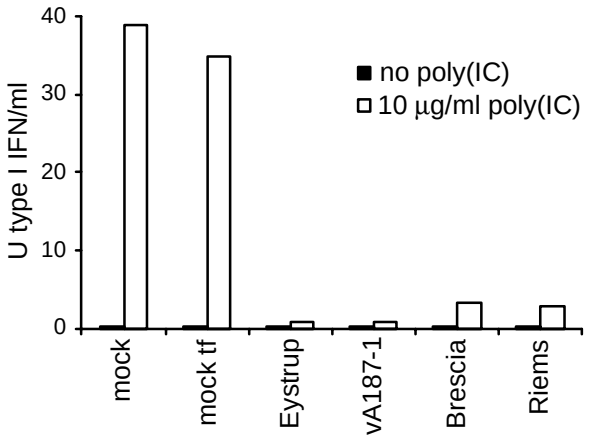

FIG. 3. CSFV interferes with poly(IC)-induced interferon production in MΦ. MΦ were infected with CSFV strains or cDNA-derived recombinant viruses as indicated on the *x* axis (MOI, 5 $TCID_{50}$/cell) or mock infected with SK-6 supernatant (mock) or with supernatant of mock-transfected SK-6 cells (mock tf) and cultured for 24 h at 38°C. Then, some cultures were left without treatment (black bars) or treated with 10 μg of poly(IC)/ml (white bars) for another 24 h. Antiviral activity in virus-inactivated cell culture supernatants was measured in PK-15 cells with the VSV antiviral bioassay and quantified based on recombinant IFN-α, as described in Materials and Methods. The detection limit was 0.5 U of IFN-α/β/ml.

pigs suggested a crucial role of N^{pro} for the expression of virulence (D. Mayer, M. A. Hofmann, and J. D. Tratschin, submitted for publication). Therefore, the growth characteristics of two independent ΔN^{pro} CSFVs, namely, vA187-ΔN^{pro} and vA187-ΔN^{pro}-Ubi, were compared in the SK-6 and PK-15 cell lines and in MΦ. In SK-6 cells, the two ΔN^{pro} CSFVs and the parent vA187-1 replicated to similar titers of approximately 10^7 $TCID_{50}$/ml within 48 h (Fig. 4A). However, analysis of the exponential growth phase of these viruses demonstrated that the replication of the ΔN^{pro} CSFV was delayed by 6 h (Fig. 4A). This delay was also observed in PK-15 cells (Fig. 4B) and MΦ (Fig. 4C), but in these cells the maximum titer for ΔN^{pro} CSFV was 1.5 to 2 $\log_{10}$ $TCID_{50}$/ml lower than the titer of the parent virus. In addition to these differences in virus titer, FCM analysis of infected PK-15 and MΦ demonstrated differences in the expression of the viral protein NS3 (Fig. 4D). For the parent virus vA187-1, 89 to 95% of the cells stained positive for NS3 after 24 h and 40 h. With the vA187-ΔN^{pro} or vA187-ΔN^{pro}-Ubi mutants, at the same MOI as the parent virus, only part of the PK-15 cells and MΦ was positive for NS3 (Fig. 4D).

ΔN^{pro} CSFV does not protect SK-6 cells from poly(IC)-induced cell death. The replication characteristics in MΦ and PK-15 cells of ΔN^{pro} CSFV compared with the parent virus pointed to a possible role of N^{pro} in CSFV escape from particular cellular antiviral defenses. In addition to producing IFN, eukaryotic cells can also respond to viral infection by entering into apoptotic cell suicide (5), which is also part of the cellular antiviral system. Wild-type CSFV can certainly interfere with the induction of both apoptosis and IFN (see above). Consequently, it was of interest to determine the influence of the presence of the N^{pro} gene on the capacity of CSFV to protect cells against poly(IC)-induced cell death.

Unlike the wild-type vA187-1, neither vA187-ΔN^{pro} nor vA187-ΔN^{pro}-Ubi could protect SK-6 cells from poly(IC)-induced cell death (Fig. 5A). The rate of poly(IC)-induced cell death of SK-6 cells infected with ΔN^{pro} CSFV was comparable to that of mock-infected cells. At the time of poly(IC) addition, parallel cultures were stained for expression of glycoprotein E2. They displayed a similar immunostaining pattern for all three viruses (Fig. 5B), in accordance with the FCM analysis of the relative NS3 expression (Fig. 4D, SK-6 cells) and with the replication characteristics of all three viruses in SK-6 cells (Fig. 4A). This confirmed that the cell cultures were equivalently infected and expressed comparable levels of viral proteins at the time of poly(IC) stimulation.

In order to determine if the requirement for N^{pro} in protection from poly(IC)-induced cell death was strain independent, poly(IC)-treated cell survival assays were repeated with a ΔN^{pro} CSFV of the highly virulent strain vEy-37 (vEy-ΔN^{pro}). Similar to the results obtained for the vA187-1-derived viruses, SK-6 cells infected with vEy-ΔN^{pro} were not protected from poly(IC)-induced cell death and behaved like the uninfected cells (data not shown).

ΔN^{pro} CSFV does not prevent poly(IC)-induced type I IFN production in MΦ. In the light of the lower levels of replication observed for the two ΔN^{pro} CSFVs in MΦ than for the wild-type virus, the capacity of ΔN^{pro} CSFV to prevent poly(IC)-induced type I IFN production was analyzed. Porcine MΦ were mock infected or infected with vA187-1, vA187-ΔN^{pro}, or vA187-ΔN^{pro}-Ubi and cultured for 24 h before poly(IC) stimulation. Similar to the results presented in Fig. 3, poly(IC)-stimulated mock-infected MΦ produced 10 to 40 U of type I IFN, whereas vA187-1-infected MΦ did not respond to poly(IC) stimulation by producing detectable type I IFN (Fig. 6). However, compared to the data obtained with the VSV assay (see Fig. 3), the detection limit was eight times higher (4 U of type I IFN/ml) when the Mx/CAT reporter gene assay was used to measure samples containing poly(IC). This was caused by IFN induction in the MDBK-t2 cells when poly(IC) was present at concentrations above 0.25 μg/ml. Nevertheless, CSFV-infected MΦ produced no or very low levels of type I IFN after poly(IC) stimulation in a total of eight independent experiments. In contrast, in five independent experiments, ΔN^{pro} CSFV-infected MΦ responded to poly(IC) with type I IFN production comparable to that of mock-infected MΦ. Two of these experiments are shown in Fig. 6. Although the level of IFN production was variable between experiments, these differences were statistically significant when a background IFN production corresponding to the level of the detection limit was used for the statistical analyses (v187-ΔN^{pro}, $P = 0.01$; vA187-ΔN^{pro}–Ubi, $P = 0.03$). Incubation of the MΦ supernatants with anti-porcine IFN-α antibody prior to addition into the Mx/CAT assay abolished the CAT response (data not shown).

ΔN^{pro} CSFV induces type I IFN in MΦ and PK-15 cells but not in SK-6 cells. In the absence of poly(IC), no detectable IFN was induced in MΦ and PK-15 cells by any of the wild-type CSFV strains tested. This contrasted with either vA187-ΔN^{pro} or vA187-ΔN^{pro}-Ubi, for which, in the absence of any poly(IC) stimulation, supernatants of infected cells contained between 1.5 and 8.5 U of type I IFN/ml for MΦ (Fig. 7A) and between 3 and 7.5 U of type I IFN/ml for PK-15 cells (Fig. 7B). For the

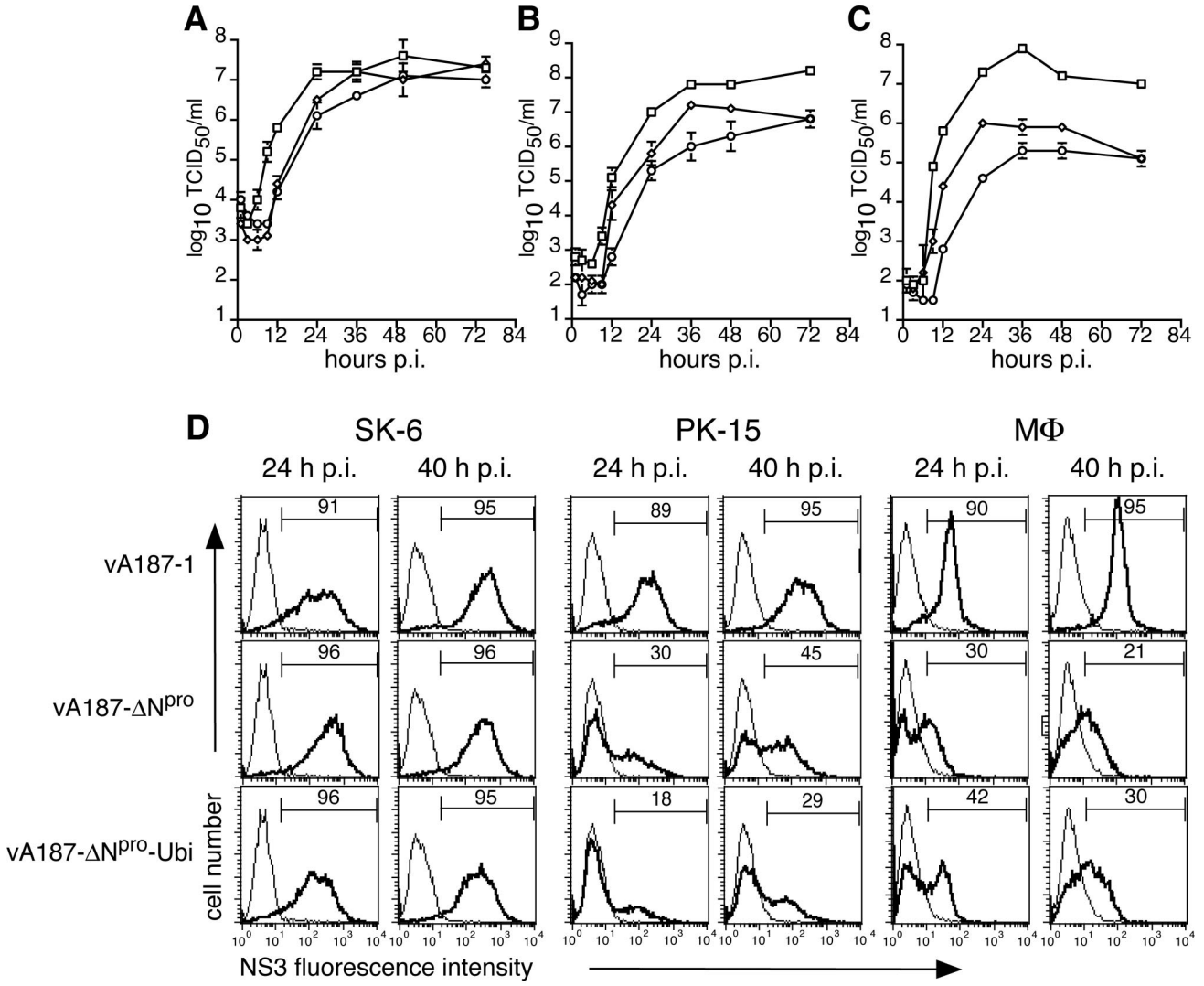

FIG. 4. Virus replication in SK-6, PK-15, and Mϕ. CSFV vA187-1 (□), vA187-ΔN^{pro} (◇), and vA187-ΔN^{pro}-Ubi (○) were used to infect SK-6 cells (A), PK-15 cells (B), and PBMC-derived MΦ (C) at an MOI of 1 $TCID_{50}$/cell for SK-6 and 5 $TCID_{50}$/cell for PK-15 and MΦ. At various times p.i., virus was harvested and the titer was determined on SK-6 cells by endpoint dilution. Each point represents the mean of results of three parallel experiments for SK-6 and PK-15 cells and two parallel experiments for MΦ, with error bars showing the standard deviation of the mean. (D) NS3 expression in SK-6, PK-15, and MΦ infected with vA187-1 and ΔN^{pro} CSFV (MOI, 5 $TCID_{50}$/cell) was analyzed 24 h and 40 h p.i. by FCM (bold lines). The light lines represent background fluorescence of NS3 staining in uninfected controls, and the percentage of NS3-positive cells is indicated for each graph. The experiment was repeated twice with similar results.

MΦ, the results of four independent experiments using MΦ preparations from separate batches of PBMC are shown, with the type I IFN quantified in the Mx/CAT reporter gene assay. The levels of type I IFN were between 5 and 25 times lower than those observed after poly(IC) stimulation of uninfected MΦ (see Fig. 3 and 6). ΔN^{pro} CSFV-infected MΦ induced statistically significantly more IFN compared to wild-type CSFV and mock-infected Mϕ, even when the calculations were based on background IFN levels at the detection limit of the assay ($P < 0.03$). Comparable results were obtained with PK-15 cells, with less variability (Fig. 7B). Interestingly, incubation of the supernatants of ΔN^{pro} CSFV-infected cells with anti-porcine IFN-α antibody prior to analysis in the Mx/CAT assay could completely block the IFN activity in the MΦ supernatants, whereas the activity in the PK-15 supernatants was only slightly reduced (data not shown). This suggests that MΦ produce mainly IFN-α, whereas PK-15 cells mainly secrete IFN-β in response to infection with ΔN^{pro} CSFV. Similar experiments were performed with supernatants from SK-6 cells infected with vA187-1, vA187-ΔN^{pro}, or vA187-ΔN^{pro}-Ubi or stimulated with poly(IC) as control. None of the supernatants harvested at 12-h intervals between 6 and 65 h p.i. revealed any detectable type I IFN activity in the Mx/CAT reporter gene assay, regardless of whether virus was inactivated or not prior to the IFN assay (data not shown). This demonstrates that SK-6 cells, in contrast to MΦ and PK-15 cells, do not produce type I IFN in response to infection with ΔN^{pro} CSFV.

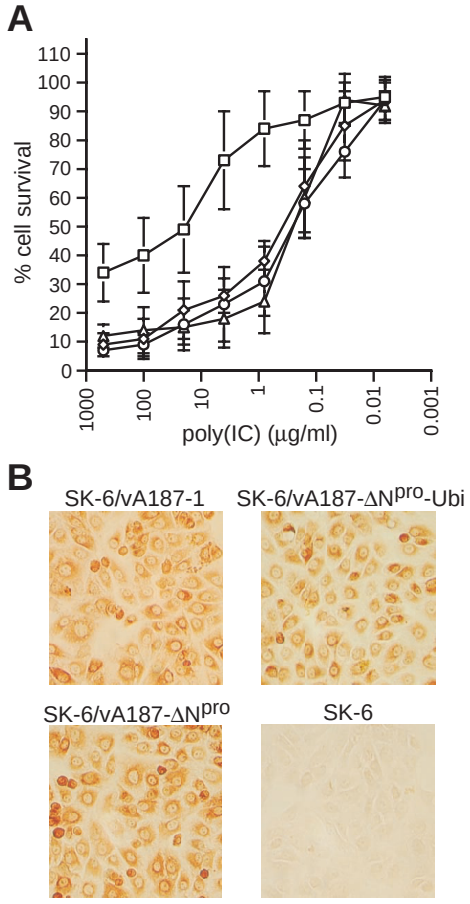

FIG. 5. ΔN^{pro} CSFV does not protect SK-6 cells from poly(IC)-induced cell death. (A) SK-6 cells were infected with CSFV vA187-1 (□), vA187-ΔN^{pro} (◇), and vA187-ΔN^{pro}-Ubi (○) or were mock infected (△) 72 h prior to treatment with poly(IC) or mock treatment. Cell survival was monitored 48 h later by crystal violet staining and measurement of OD_{595}. The percentage of cell survival was calculated from the ratio of the OD_{595} of poly(IC)-treated wells to that of mock-treated wells, in analogy to the data shown in Fig. 1. The mean values with standard deviation from four independent experiments are shown for decreasing poly(IC) concentrations. (B) At the time of poly(IC) addition, parallel cultures were analyzed for expression of the viral glycoprotein E2 using immunoperoxidase staining.

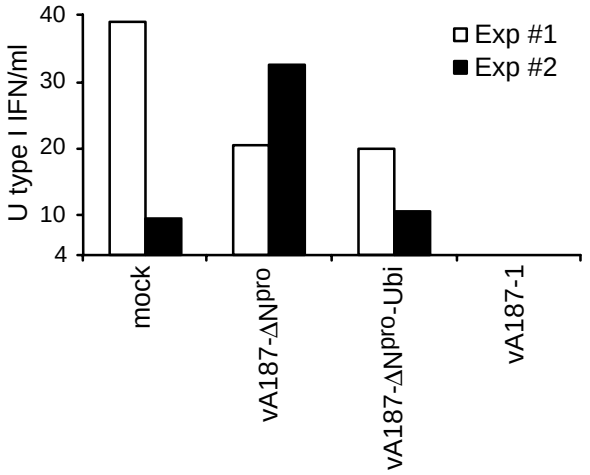

FIG. 6. ΔN^{pro} CSFV does not inhibit poly(IC)-induced type I IFN synthesis in MΦ. MΦ were mock infected or infected at an MOI of 5 $TCID_{50}$/cell with either of the two ΔN^{pro} CSFVs or with the parent vA187-1, as indicated on the *x* axis, and cultured for 24 h. Then, the cultures were treated with 10 μg of poly(IC)/ml for 24 h in the first experiment (white bars) and 40 h in the second, independent experiment shown (black bars). IFN-α/β activity was measured in cell culture supernatants using the Mx/CAT reporter gene assay and recombinant porcine IFN-α for quantitation. The detection limit is given by the lower limit of the *y* axis (4 U of type I IFN/ml).

ΔN^{pro} CSFV interferes with VSV replication in PK-15 cells. Supernatants from MΦ infected with ΔN^{pro} CSFV, as opposed to supernatants from wild-type CSFV-infected MΦ, induced a clear antiviral activity against VSV in PK-15 cells. This activity could be neutralized with anti-IFN-α antibody only if the infectious CSFV in the MΦ supernatants was inactivated (data not shown). This indicated that in contrast to wild-type virus, ΔN^{pro} CSFV could interfere directly with VSV replication upon coinfection of PK-15 cells. In order to confirm this hypothesis, PK-15 cells were infected with 10-fold dilutions of either vA187-1, vA187-ΔN^{pro}, or vA187-ΔN^{pro}-Ubi produced in SK-6 cells, starting with an MOI of 10 $TCID_{50}$/cell. As mentioned above, supernatants of SK-6 cells infected with either wild-type or ΔN^{pro} CSFV do not contain type I IFN activity, as determined in the Mx/CAT reporter gene assay. After 24 h, the CSFV-infected PK-15 cell cultures were superinfected with VSV at an MOI of 5 $TCID_{50}$/cell, a dose resulting in complete cpe in uninfected control cells within 24 h. As opposed to vA187-1, both ΔN^{pro} CSFVs protected PK-15 cells from the VSV-induced cpe in a dose-dependent manner (Fig. 8A). This protection was dependent on the presence of infectious virus, since preincubation of the CSFV inocula with a virus-neutralizing dose of anti-E2 MAb abolished the interference with VSV-induced cpe (Fig. 8B). Preincubation of the CSFV inocula with a neutralizing dose of anti-IFN-α antibody, however, did not reverse the interference effect (Fig. 8C). At the time of VSV challenge, a duplicate control culture treated as above was immunostained for CSFV E2 expression. The percentage of infected cells was dependent on the MOI, and while it was reduced by the anti-E2 MAb, there was no effect of the anti-IFN-α antibodies (data not shown). Taken together, these results suggest that CSFV lacking the N^{pro} gene, as opposed to wild-type virus, is capable of interfering with VSV replication in PK-15 cells. The presence of the N^{pro} gene in wild-type CSFV is therefore required for preventing the induction of an antiviral response in CSFV-infected cells.

DISCUSSION

The autoproteinase N^{pro} is the first protein expressed from the single large ORF of all pestiviruses (43, 47, 56) and has no counterpart within the other genera of the family *Flaviviridae* (55). When CSFV, BVDV, and BDV are compared, the amino acid sequence identity of N^{pro} is higher than 70% (40) and the residues Glu_{22}, His_{49}, and Cys_{69}, essential for the proteolytic activity of N^{pro} (43), as well as the residues Cys_{168} and Ser_{169} surrounding the cleavage site (47), are conserved. Tratschin et al. demonstrated earlier that N^{pro} of CSFV was dispensable for virus replication in cell culture (52). Nevertheless, it has been

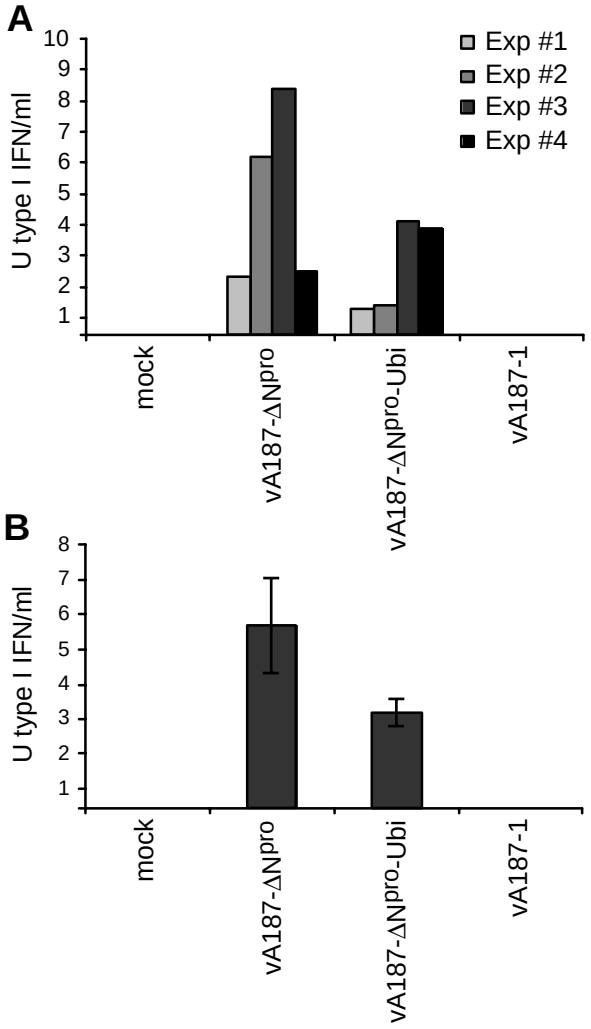

FIG. 7. ΔN^{pro} CSFV induces type I IFN synthesis in MΦ and PK-15 cells. MΦ (A) and PK-15 cells (B) were mock-infected or infected at an MOI of 5 $TCID_{50}$/cell with either of the two ΔN^{pro} CSFVs or with the parent vA187-1, as indicated on the *x* axis. The results of four independent experiments are shown. For MΦ, the samples were harvested and analyzed at 24, 36, 40, and 48 h p.i. (Exp. #1 to 4, respectively). For PK-15 cells, the samples were analyzed at 40 h p.i. Type I IFN activity was measured in cell culture supernatants using the Mx/CAT reporter gene assay and recombinant porcine IFN-α for quantitation. The detection limit is given by the lower limit of the *y* axis (0.5 U of type I IFN/ml).

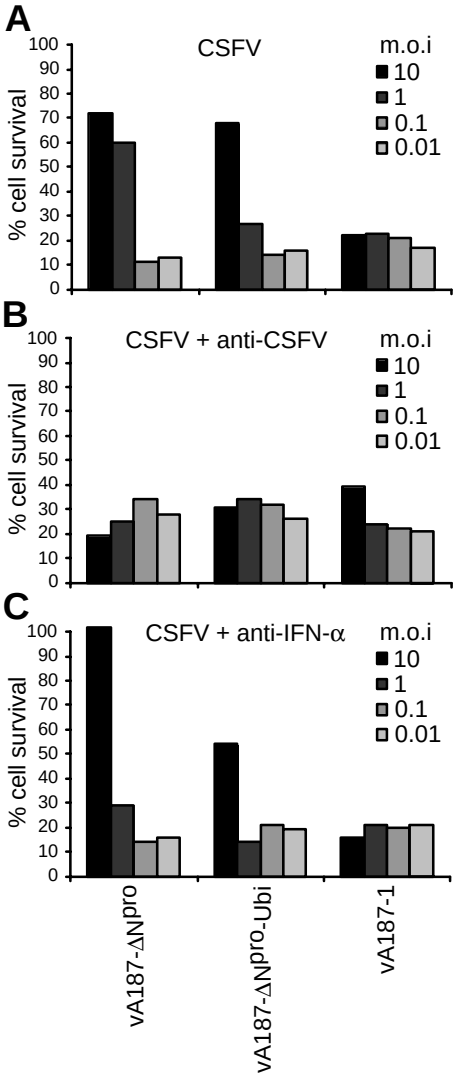

FIG. 8. ΔN^{pro} CSFV interferes with VSV replication in PK-15 cells. PK-15 cells were infected at an MOI of 10, 1, 0.1 and 0.01 $TCID_{50}$/cell with ΔN^{pro} CSFV and with the corresponding parent vA187-1 as indicated. Cells were cultured for 24 h prior to superinfection with VSV at an MOI of 5 $TCID_{50}$/cell, resulting in complete cpe within 24 h in uninfected controls. Twenty-four hours after VSV infection, cell survival was monitored by crystal violet staining and measurement of OD_{595}. The experiments were performed in the absence (A) or presence (B) of a neutralizing titer of MAb HC/TC26 or in the presence of polyclonal anti-IFN-α serum (C). Results from a representative experiment out of three are shown.

retained during evolution. Therefore it was tempting to postulate that N^{pro} exerted additional functions in vivo besides simply cleaving itself off the nascent polyprotein. The first piece of evidence was obtained with the observation that the lack of N^{pro} completely abolished the virulence of the moderately virulent strain vA187-1 (52) and of the highly virulent strain vEy-37 (D. Mayer, M. A. Hofmann, and J. D. Tratschin, submitted for publication).

In the present study, we analyzed the interaction of CSFV with cellular antiviral defense in vitro. For this purpose, we used cell stimulation with the synthetic dsRNA macromolecule poly(IC), a well-known biological response modifier, as a model for activation of nonspecific antiviral activities, i.e., apoptosis in the porcine SK-6 cell line and type I IFN induction in porcine monocytic cells. It was shown earlier that preinfection of cells with BVDV suppressed the poly(IC)-induced interference with VSV replication and cpe, a characteristic exploited in a diagnostic assay for the detection of BVDV (33). Rossi and Kiesel, however, suggested that the poly(IC)-induced interference with VSV was a non-IFN-based mechanism (41). More recent data published by Schweizer and Peterhans clearly demonstrated that BVDV blocked poly(IC)-induced type I IFN transcription and secretion (44). In addition, BVDV completely protected bovine turbinate cells from poly(IC)-in-

duced apoptosis. A very recent study reported similar observations for hepatitis A virus that was also shown to inhibit poly(IC)-induced IFN-β synthesis and apoptosis (8). For both BVDV and CSFV, the interference with antiviral activities was recognized a long time ago with the observation that these viruses enhanced the multiplication of Newcastle disease (ND) virus in cell culture (24, 31). This phenomenon was designated END (for "exaltation of ND virus") and applied to a quantitative assay for BVDV (24) and CSFV (30). More recently, BVDV was also shown to enhance replication of orbiviruses (38). It was suggested that the mechanism of END was a result of suppression of IFN production (13, 51). The results presented herein demonstrate that CSFV, by analogy to BVDV, is capable of preventing poly(IC)-mediated induction of cellular antiviral activities. Together with the reports mentioned above, our data indicate that the interference with cellular antiviral defense might be a general feature of most pestivirus strains. It is therefore not surprising that for both BVDV (44) and CSFV (this study), the inhibition of IFN induction was independent of the origin and virulence of the virus strain analyzed. Interestingly, Shimizu and coworkers reported an attenuated CSFV strain that did not suppress IFN production and was itself an effective IFN inducer (46, 54). This strain had been attenuated by serial passage in pigs followed by multiple passages in swine and bovine testicle cells. Finally, after 30 passages in guinea pig cells, a clone inducing interference with ND virus was isolated and designated GPE^- (END-negative). The CSFV-mediated inhibition effect on the heterologous interference caused by the CSFV strain GPE^- against VSV replication in cell culture was exploited in a diagnostic assay named reverse interference method (54).

The phenotype of the strain GPE^- in cell culture is reminiscent of the characteristics we observed with the CSFV mutants lacking the N^{pro} gene. In contrast to the wild-type parent virus, ΔN^{pro} CSFV did not suppress poly(IC)-induced type I IFN production in MΦ and apoptosis in SK-6 cells, indicating a crucial role for N^{pro} in the interference of the virus with antiviral pathways.

It is important to note that after infection with either vA187-1 or ΔN^{pro} CSFV, all SK-6 cells were infected and expressed comparable amounts of glycoprotein E2 and of nonstructural protein NS3 at the time of poly(IC) addition. This indicates that the loss of the capacity of ΔN^{pro} CSFV to interfere with poly(IC)-induced apoptosis is not associated with reduced viral replication or protein synthesis. Nevertheless, the fact that only part of the MΦ population expressed detectable virus antigen when infected with ΔN^{pro} CSFV has to be taken into account when interpreting the regained poly(IC) responsiveness of MΦ cultures infected with ΔN^{pro} CSFV compared with wild-type virus. Nevertheless, the role of N^{pro} in the inhibition of antiviral processes was substantiated by the observation that CSFV lacking N^{pro} actively induced type I IFN in MΦ and PK-15 cells as well as resistance to VSV-induced cpe in PK-15 cells. The latter could be mediated in part through the activation of intrinsic antiviral activity. As a consequence of the ΔN^{pro} CSFV-mediated antiviral activity, part of the MΦ and PK-15 population probably becomes resistant to CSFV replication and might still respond to poly(IC). Thus, the observation that ΔN^{pro} CSFV does not prevent poly(IC)-induced type I IFN production in MΦ could be the result of two overlapping mechanisms, namely, the loss of an inhibitory activity of N^{pro} on type I IFN production and the reduced viral replication due to the induction of an antiviral state in the MΦ culture.

Although wild-type CSFV does not have the capacity to induce IFN in MΦ, we had previously shown that replication of CSFV was inhibited by pretreatment of the cells with recombinant porcine IFN-α, resulting in a virus titer reduction of 2 $\log_{10}$ $TCID_{50}$/ml (28). The IFN-mediated titer reduction correlates with the impaired replication observed in MΦ and PK-15 cells with ΔN^{pro} CSFV compared to vA187-1. It has been observed repeatedly that viral genes that were dispensable for virus replication in cell cultures or in mice deficient in a defined antiviral activity were essential to counteract this antiviral activity in the wild-type parent animal or cell. Some examples are NS1 of influenza A virus (16); NSs of Bunyamwera virus (9) and Rift Valley fever virus (7); NS1, NS2, SH, and M2-2 of respiratory syncytial virus (10, 26); the V protein of simian virus 5 (22); and the L-proteinase of foot-and-mouth disease virus (11).

The active induction of antiviral processes in MΦ and PK-15 cells by mutant CSFV lacking the N^{pro} gene argues against the possibility that ΔN^{pro} CSFVs simply have lost their capability to interfere with poly(IC)-induced antiviral activities because of a possible reduced replication level as a result of altered translation initiation due to the modifications of the RNA structures at the boundary between the internal ribosomal entry site and the ORF. Furthermore, the hypothesis that N^{pro} is required for direct interference of CSFV with endogenous antiviral pathways is supported by the data of N^{pro}-dependent resistance to poly(IC)-induced apoptosis in SK-6 cells.

A central starting point in the complex cascade of signal transduction events involved in the endogenous cellular antiviral defense is the appearance of intracellular dsRNA, which is provided by the viral genome itself or formed during replication and transcription of viral genomes (25). The best-characterized pathway induced by viral dsRNA involves activation of the dsRNA-dependent protein kinase (PKR) and of 2′-5′ oligoadenylate synthetase which is followed by activation of eIF-2α and RNaseL, respectively, both leading to apoptosis (for selected reviews, see references 12, 17, and 18). A second important dsRNA-dependent pathway induces type I IFN and other cytokines and chemokines through activation of PKR and NF-κB (4). Type I IFN induction by viruses and dsRNA is also dependent on activation and nuclear translocation of the interferon regulatory factor (IRF) family of transcription factors (reviewed in reference 50). Interestingly, it was shown recently that ncp BVDV induced translocation of IRF-3 into the nucleus without subsequent binding to DNA. Furthermore, ncp BVDV was able to block Semliki Forest virus-induced IFN-β production through a block in the formation of IRF-3–DNA complexes (3). Whether this is also true for CSFV and whether N^{pro} is involved in this process remain to be investigated. A third antiviral pathway involves the IFN-inducible Mx proteins, which are large GTPases with homology to dynamin (21, 23). Nevertheless, the remaining innate resistance of triple-deficient RNaseL, PKR, and Mx1 mice to virus infections indicates the presence of additional pathways (57).

During evolution, viruses have learned to interfere with most if not all of these complex antiviral systems (for selected

reviews, see references 1, 5, 15, 18, and 45). Although the cellular pathway with which N^{pro} might interact remains to be characterized, our data suggest a novel function of N^{pro} in counteraction of cellular antiviral defense. The importance of this function of N^{pro} becomes lucent when considering the pathogenesis of CSF. Only the presence of N^{pro} permits efficient infection of monocytic cells, including monocytes, MΦ, and even dendritic cells (data not shown). These cells are among the main targets for CSFV, allow high-level replication, and permit cell-associated spreading and colonization of immunological tissue by CSFV. Furthermore, they appear to play a central role in virus-induced immunomodulation (29). Interestingly, ΔN^{pro} CSFV of both the moderately virulent strain Alfort/187 and the highly virulent strain Eystrup were attenuated in pigs (D. Mayer, M. A. Hofmann, and J. D. Tratschin, submitted for publication). The relationship of this finding with the in vitro data presented here, as well as the role of N^{pro} in the pathogenesis of CSF, remains to be established.

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