ORF7-encoded accessory protein 7a of feline infectious peritonitis virus as a counteragent against IFN-α-induced antiviral response


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The type I IFN-mediated immune response is the first line of antiviral defence. Coronaviruses, like many other viruses, have evolved mechanisms to evade this innate response, ensuring their survival. Several coronavirus accessory genes play a central role in these pathways, but for feline coronaviruses this has never to our knowledge been studied. As it has been demonstrated previously that ORF7 is essential for efficient replication in vitro and virulence in vivo of feline infectious peritonitis virus (FIPV), the role of this ORF in the evasion of the IFN-α antiviral response was investigated. Deletion of ORF7 from FIPV strain 79-1146 (FIPV-Δ7) rendered the virus more susceptible to IFN-α treatment. Given that ORF7 encodes two proteins, 7a and 7b, it was further explored which of these proteins is active in this mechanism. Providing 7a protein in trans rescued the mutant FIPV-Δ7 from IFN sensitivity, which was not achieved by addition of 7b protein. Nevertheless, addition of protein 7a to FIPV-Δ3Δ7, a FIPV mutant deleted in both ORF3 and ORF7, could no longer increase the replication capacity of this mutant in the presence of IFN. These results indicate that FIPV 7a protein is a type I IFN antagonist and protects the virus from the antiviral state induced by IFN, but it needs the presence of ORF3-encoded proteins to exert its antagonistic function.

INTRODUCTION

Coronaviruses (CoVs) generally cause mild diseases during respiratory and intestinal infections in mammals and birds, but may exceptionally lead to life-threatening diseases, such as severe acute respiratory syndrome (SARS) or Middle East respiratory syndrome in humans, and feline infectious peritonitis (FIP) in felids (Drosten et al., 2003; Ward et al., 1968; Zaki et al., 2012). Despite their economical and sentimental importance, information on the molecular mechanisms governing CoV virulence and pathogenesis is scarce. Feline infectious peritonitis virus (FIPV), which is the causal agent of the fatal systemic disease FIP in cats, is one of the CoVs for which significant research efforts still have not resulted in a fully elucidated pathogenesis. FIPV shares high genetic and structural similarity with feline enteric coronavirus (FECV), which causes at most a temperate self-limiting diarrhoea as a result of a mild enteritis evoked by replication of the virus in enterocytes (Hayashi et al., 1982; Herrewegh et al., 1997; Kipar et al., 2010; Meli et al., 2004; Pedersen et al., 1981). It has been shown that mutation from FECV to FIPV within an infected cat is the most probable means of acquiring FIP (Chang et al., 2010; Herrewegh et al., 1995; Licitra et al., 2013; Myrrha et al., 2011; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). The causal mutations will favour replication of the virus inside blood monocytes, causing a systemic spread of FIPV, resulting in an immune-mediated vasculitis and ultimately leading to death (Dewerchin et al., 2005; Rottier et al., 2005; Stoddart & Scott, 1989; Vennema et al., 1998). Although any cat that carries FECV is potentially at risk of developing FIP, it is typically seen that cats with an ineffective immune system are more likely to develop the disease (Addie et al., 1995).

Like all viruses, FIPV is an intracellular parasite of its host cell. Viral replication and transcription occur in the cytoplasm, leading not only to new genome molecules and the typical nested set of subgenomic mRNAs but also to dsRNA intermediates (Enjuanes et al., 2006; Gorbalenya et al., 2006; Spaan et al., 1988). Furthermore, FIPV does not encode the enzymic machinery to translate its subgenomic mRNAs. Therefore, it is obliged to make use of the cellular apparatus for protein synthesis (Thompson & Sarnow, 2000). This dependency, together with the presence of viral dsRNA species, enables the invaded host cell to recognize the viral intruder and triggers diverse immune responses (Medzhitov & Janeway, 1997; Takeuchi et al., 1995).
& Akira, 2009). The secretion of type I IFNs, IFN-α and IFN-β, is the anchor of the innate host defence against virus infection. This drive both autocrine and paracrine signalling, which induces IFN-stimulated gene (ISG) products with antiviral activities, resulting for instance in a global protein synthesis arrest. Because of the crucial importance of ensuring translation of mRNA, viruses have evolved strategies to breach these cellular defences (Gale & Sen, 2009; Grandvaux et al., 2002; Randall & Goodbourn, 2008; Taylor & Mossman, 2013). In general, CoVs express an impressive variety of viral proteins that act as modulators of the IFN gateway. Viral evasion proteins can degrade or inhibit IFN transcription factors or antagonize IFN signalling [nsp1 of SARS-CoV, protein 5a of mouse hepatitis virus (MHV)], as well as hijack cellular pathways or modulate ISG products [N protein of MHV, nsp2 of infectious bronchitis virus (IBV) and protein 7 of transmissible gastro-enteritis virus (TGEV)] (Cruz et al., 2011; Kamitani et al., 2006; Koetzen et al., 2010; Wang et al., 2009; Ye et al., 2007). For FIPV, not much is known about the battle between the body’s defence mechanisms and the invading virus, but it seems likely that FIP only occurs if the virus has some means of circumventing the IFN response. In this respect, accessory proteins have been pointed out to be of key importance for virus-host interactions and, given the impaired replication of FIPV deleted in the accessory ORF7 (Dedeurwaerder et al., 2013) and the attenuation of these mutant viruses in vivo (Haijema et al., 2004), proteins encoded by this ORF deserve further attention. ORF7 encodes two proteins. Protein 7a is a small hydrophobic protein of 101 aa (~10 kDa) with an N-terminal cleavable signal sequence and a C-terminal transmembrane domain (Thiel, 2007). It has 72% sequence homology with protein 7 of TGEV (Cruz et al., 2011). Protein 7b is a soluble glycoprotein of ~24 kDa (207 aa), from which expression in natural infections has already been implied (Kennedy et al., 2008). It contains a KDEL-like endoplasmic reticulum (ER) retention signal (KTEL) at its C terminus (Vennema et al., 1992). This allows initial ER attachment, whereupon it is secreted from the infected cell.

In this study, it was investigated whether the hampered replication seen for ORF7 deletion mutants of FIPV could be explained by a higher susceptibility of these mutants for IFN-α and which proteins encoded by ORF7 were involved in this mechanism.

RESULTS

In vitro and in vivo IFN-α production during feline CoV (FCoV) infection

Feline IFN-α was produced in the supernatants of Felis catus whole fetus (fcwf-4) cells infected with FIPV-wt and FIPV-Δ7 at m.o.i. of 0.02, as detected by an IFN-α bioassay. The data are presented in Table 1. No significant difference was observed between the wt virus and its mutant. In addition, no significant differences in IFN-α production were detected with infected cells expressing protein 7a or 7b (data not shown).

In the sera of cats diagnosed with FIP, a significantly higher amount of IFN-α was detected (482–8571 U ml⁻¹) than in the sera of conventional FECV-negative healthy cats, in which up to 25 times less IFN-α (154–322 U ml⁻¹) was found (Table 2). The IFN-α expression in the sera of FECV-positive cats also revealed an elevated production level in comparison with the FECV-negative cats. The sera of a cat (Baghera) experimentally infected with FECV strain UCD, revealed that IFN-α levels were remarkably elevated in early stages post-inoculation.

FIPV-wt is more resistant to IFN-α pre-treatment than its accessory ORF7 deletion mutant FIPV-Δ7

As shown in Fig. 1, FIPV-wt was sensitive to IFN treatment, typically showing an approximately 10-fold inhibition of virus production and a reduction of N protein-positive cells of about 50% at the highest dose tested (1000 U ml⁻¹). Notwithstanding, both the amount of nucleocapsid (N) expressing cells and virus titres were significantly even more reduced for FIPV-Δ7 after treatment with as little as 10 U ml⁻¹ IFN-α. At the highest dose of feline IFN-α (fIFN-α), the drop in infectious titre was of the order of 100-fold and there was a reduction in N protein-positive cells of 75%.

Localization of proteins 7a and 7b in fcwf-4 cells

In order to assess whether the absence of 7a and/or 7b protein during FIPV infection was responsible for the higher susceptibility to fIFN-α, fcwf-4 cells stably expressing FIPV 7a or 7b protein were generated. In order to detect 7a/7b protein, a GFP tag was inserted at the C terminus of 7a/7b protein, fcwf-4 cells stably expressing the GFP tag alone were used as control cells for the experiments. Expression was confirmed by Western blot and immunofluorescence. The protein bands detected by Western blot analysis proved that the accessory proteins were fused to GFP (Fig. 2). We detected the expected shift in protein size with reference to GFP alone, given that the molecular mass of the 7a protein is around 10 kDa and that of 7b protein is around 24 kDa (Thiel, 2007). As shown by fluorescence microscopy, 7a–GFP was present in the cytoplasm, co-localizing mainly with ER and the Golgi apparatus; 7b–GFP was targeted to the perinuclear region (Golgi apparatus), from where it dispersed in the cytoplasm as dense spots, to eventually reach the cell membrane (Fig. 3). In contrast to the 7a– and 7b–GFP fusion proteins, GFP alone was present in all cell compartments. This indicated the accessory proteins determine the localization of their GFP fusion proteins.

Protein 7a but not 7b restores the inhibitory effect of fIFN-α on FIPV-Δ7 replication

The effect of 7a or 7b protein provided in trans to FIPV-Δ7 on fIFN-α susceptibility was analysed. FIPV-Δ7 infection of
Table 1. IFN-α measurements in the supernatant of FIPV-wt- and FIPV-Δ7-infected fcwf-4 cells (m.o.i. of 0.02), determined by an IFN bioassay

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>IFN-α titre (U ml⁻¹)</th>
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<tr>
<td></td>
<td>Replication 1</td>
</tr>
<tr>
<td>FIPV-wt 18 h p.i. fcwf-4</td>
<td>100</td>
</tr>
<tr>
<td>FIPV-Δ7 18 h p.i. fcwf-4</td>
<td>33</td>
</tr>
<tr>
<td>FIPV-wt 24 h p.i. fcwf-4</td>
<td>870</td>
</tr>
<tr>
<td>FIPV-Δ7 24 h p.i. fcwf-4</td>
<td>648</td>
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fcwf-4 cells expressing GFP alone was used as control, excluding the effect of GFP on infection.

As previously observed with the untransduced fcwf-4 cells, there was a significant reduction of FIPV-Δ7 replication in the fcwf–GFP cells after fIFN-α treatment (80% reduction in N protein-positive cells and 200-fold drop in infectious titre). Under this fIFN-α pressure, 7a–GFP, but not 7b–GFP, had the capacity to significantly elevate N protein expression (7a–GFP: \( P = 0.0286 \) for all three IFN concentrations; 7b–GFP: \( P = 0.3429 \), 0.4429 and 0.3858 for 10, 100 and 1000 U IFN, respectively) and virus production (7a–GFP: \( P = 0.0286 \) for all three IFN concentrations; 7b–GFP: \( P = 0.3429 \), 0.4429 and 0.3858 for 10, 100 and 1000 U IFN, respectively) of FIPV-Δ7 in comparison with GFP alone (Fig. 4).

In the presence of protein 7a, the reduction in viral N-producing cells and virus production was only 50% and 14-fold, respectively, at the highest dose of IFN-α, which was comparable with the IFN sensitivity of FIPV-wt.

Table 2. IFN-α measurements in cat sera and FCoV diagnosis

Diagnosis of FECV-infected cats was determined by immunoperoxidase monolayer assay (IPMA), detecting FCoV antibodies in the serum, and by real-time reverse transcriptase-PCR (optimized in our laboratory; Desmarets et al., 2013), detecting FCoV RNA in the faeces. Yana was transiently infected at the moment of sample analysis and resolved the infection (no remaining faecal shedding). Chablis appeared to be persistently infected with FECV as he was still shedding virus in his faeces (at follow up from October 2011). Baghera was a specific-pathogen-free cat that was orally inoculated with FECV strain UCD (Vermeulen, 2013). His faeces and sera were analysed at 5 and 28 days p.i. No viral copies were detected in the faeces from 56 days p.i. FIP was verified in sick cats by post-mortem diagnosis and presence of FCoV N protein expression in cells purified from ascites and in FIP lesions.
At low IFN-α levels, 7b–GFP had some positive effect on the amount of cells expressing viral N protein, but this did not reach a significant level \( (P=0.1714) \). Providing 7a–GFP or 7b–GFP \textit{in trans} to FIPV-wt induced no significant increase in titre or viral N protein-expressing cells under IFN pressure \( (P>0.3) \).

**Protein 7a cannot restore vesicular stomatitis virus (VSV) sensitivity towards IFN-α**

Previous results emphasized the important role of protein 7a during IFN-α antagonism, and it was our purpose to further evaluate its mechanism. Therefore, it was examined if the protein could function on its own or if it needed the expression of other FCoV-encoded proteins. To investigate this, protein 7a was provided \textit{in trans} to a highly IFN-sensitive VSV virus and the effect on replication under IFN-α pressure was analysed. As shown by viral TCID\(_{50}\), VSV replication was hampered to the same amount in the control cells as in the cells expressing protein 7a at all IFN concentrations tested \( (P=0.35\) at 10 U IFN-α and \( P=0.5\) at 100 and 1000 U IFN-α) (data not shown). Thus, the antagonistic function of protein 7a was not able to reduce the sensitivity of another IFN-sensitive virus like VSV. Moreover, it should be noted that VSV was far more sensitive to IFN treatment than FIPV-wt, with more than 100 times reduction of viral titre at the lowest IFN dose (10 U) tested and 1000 times less viral growth in the presence of 1000 U IFN-α.

**FIPV-Δ3 and FIPV-Δ3Δ7 replication is less efficient than that of FIPV-wt after IFN-α treatment**

The fact that protein 7a could not exert its function on a virus other than FIPV led to the hypothesis that protein 7a might need the expression of other FCoV-encoded proteins to exert its function. As two other mutants of FIPV, one deleted in ORF3 (FIPV-Δ3) and one deleted in both accessory ORFs (FIPV-Δ3Δ7), were also attenuated \textit{in vivo} (Dedeurwaerder et al., 2013; Haijema et al., 2004), it was of interest to investigate their sensitivity to IFN-α. Fig. 5 shows that these two accessory protein mutants of FIPV encountered a negative effect on their replication after IFN-α treatment similar to that demonstrated for FIPV-Δ7 \( (P=0.05\) in comparison with FIPV-wt, N expression as TCID\(_{50}\) results, at all IFN concentrations tested). This could explain their similar attenuated behaviour \textit{in vivo}.

**Protein 7a cannot reverse the inhibitory effect of IFN-α on FIPV-Δ3Δ7 infection**

The results described above, specifically that (i) protein 7a needs the expression of other FCoV proteins and (ii)
FIPV-Δ3 was as sensitive to IFN treatment as FIPV-Δ7, indicating that 7a is not able to completely antagonize the IFN-induced antiviral response without the presence of ORF3. This could be further evaluated by investigating the effect of providing 7a \textit{in trans} to FIPV-Δ3Δ7. As shown in Fig. 6, the presence of 7a did not significantly increase the replication efficiency of FIPV-Δ3Δ7 in comparison with GFP ($P \geq 0.2$). Only at the highest IFN dose did 7a protein induce a significant ($P=0.05$) increase in viral titre (Fig. 6b), but still protein 7a could not induce levels of FIPV-wt similar to those of FIPV-wt under this IFN pressure. In general, protein 7a could not restore the replication efficiency of FIPV-Δ3Δ7 to the phenotype of FIPV-wt as it could for FIPV-Δ7, confirming the synergistic collaboration between 7a protein and ORF3-encoded protein(s) targeting the IFN-induced antiviral response.

**DISCUSSION**

Circumventing the early innate immune response, characterized by IFN induction, is a critical step for viruses to establish \textit{in vivo} infections. All classes of viruses encode proteins that act as modulators of one or more steps of the IFN signalling pathways (Gale & Sen, 2009). Among viruses, RNA viruses are the best inducers of IFN,
consequently leading to the discovery of an impressive array of mechanisms used by these viruses to surmount the IFN-induced antiviral pathway. In the last decade, some of these viral IFN antagonizers, often accessory proteins, have also been identified in coronaviruses. These proteins either are involved in the inhibition of IFN synthesis (such as ORF3b, ORF6 and N protein of SARS-CoV; Kopecky-Bromberg et al., 2007) or circumvent the IFN signalling pathway (such as ORF7 protein of TGEV, ORF5a and N proteins of MHV and ORF3b, ORF6 and 7a protein of SARS-CoV) (Cruz et al., 2011; Koetzner et al., 2010; Kopecky-Bromberg et al., 2006; Ye et al., 2007). To date, there has been no description of an IFN evader for FCoVs. However, research results from the last decade have led us to believe that FCoVs also counteract the IFN-induced antiviral pathway. It has been suggested many times before that ORF7 could play a role during virus–host interactions. Nevertheless, no specific function has been assigned to either of the proteins encoded by this ORF before (Kennedy et al., 2001, 2008; Lin et al., 2009). Both impaired replication of FIPV deleted in the accessory ORF7 (Dedeurwaerder et al., 2013), and attenuation of this mutant virus in vivo (Haijema et al., 2004), as well as the close genetic similarity with the 7 protein of TGEV (Cruz et al., 2011), support the hypothesis that proteins encoded by ORF7 are involved in an IFN evasion mechanism. We demonstrated that FIPV infection induced IFN-α, both in vitro and in vivo. The in vitro IFN-α sensitivity assays showed that FIPV countermeasures are not absolute as FIPV-wt also encountered replication restriction due to IFN-α pre-treatment. This supports the typical chronic interplay between FCoV and its host, as replication in vivo is mostly restricted to a low percentage of cells and the virus can persist for a couple of months without inducing any symptoms. Complete IFN activity disruption would
FIPV 7a protein is a type I IFN antagonist

also not be beneficial for the virus, as cells could interpret this as a stress signal, activating cellular apoptosis and destroying the cell before virus assembly has occurred. Notwithstanding the significant amount of IFN production in vivo and in vitro, it cannot restrict the virus replication enough to prevent a fatal disease outcome. This can also explain why IFN-α administration to FIP cats is not sufficient as a treatment (Weiss et al., 1990). This observation indicates the presence of FIPV-encoded proteins (like 7a and/or 7b, for example) that block IFN actions downstream of IFN synthesis.

In this article, we have shown that IFN-α markedly impaired the replication of FIPV with a deleted ORF7 (FIPV-Δ7) compared with FIPV-wt. In addition, this can be fully complemented by providing 7a accessory protein of the FIPV-wt, suggesting that the 7a accessory protein functions as an agent that interferes with the innate IFN-α-mediated immune response. The presence of protein 7a in advance of infection could not reduce IFN-α production by FIPV (data not shown), which indicates that 7a protein antagonizes the downstream IFN production cascade. Further evaluation of its mechanism showed that protein 7a could only interfere with the IFN-α antiviral response in the presence of one or more proteins encoded by ORF3 (3a or 3b). This conclusion was drawn from the facts that (i) protein 7a could not restore the IFN-α sensitivity of FIPV-Δ3Δ7 or VSV and (ii) all accessory ORF mutants of FIPV experienced a similar negative effect on IFN-α treatment. The synergism of protein 7a and ORF3 allows efficient replication of FIPV-wt. Whether this cooperation is characterized by a direct interaction of these proteins has to be further investigated. On the other hand, it is also possible that they function on different IFN-induced pathways which result in the same antiviral effect, for instance, inhibition of protein synthesis. Blocking both of these IFN-induced pathways could be essential for the virus to overcome the overall negative effect of IFN.

Deleting accessary ORFs from FIPV-wt did not render this mutant fully susceptible to IFN, revealing that other viral proteins (nsps and/or structural proteins) can also participate in the IFN antagonism.

The fact that 7b protein is not able to restore virus infectivity under IFN-α pressure indicates that this accessory protein fulfils its function in another pathway. The expression pattern of 7b could represent the slow export of the protein by microsomes, as suggested by Vennema et al. (1992), indicating a role in the extracellular environment of infected target cells. Given the sequence distribution of ORF7, it is not surprising that 7b protein plays a minor role compared with 7a protein in defence against first-line antiviral actions of infected cells. As 7b is translated from ORF7 by ribosomal leaky scanning, it is very likely that protein expression levels are low in comparison with 7a. Moreover, the sequence of the 7a gene is far more conserved between patho- (FIPV and FECV) and serotypes (type I and II), than that of the 7b gene, underlining the importance of the 7a protein.

This IFN-α evasion can be added to the list of earlier described immune evasion strategies of FIPV, like retention, internalization, complement blockage and suppression of lymphocyte proliferation (Cornelissen et al., 2007, 2009; Dewerchin et al., 2008; Vermeulen et al., 2013).

It has been demonstrated before that FIPV-Δ7 replication was impaired in peripheral blood monocytes, as well as in fcwf-4 cells, but not in the Crandell Rees feline kidney (CrFK) cell line (Dedeurwaerder et al., 2013). Performing the IFN-α bioassay on fcwf-4 and CrFK cells revealed also that VSV replication in CrFK cells was far less sensitive to fIFN-α pre-treatment than that in fcwf-4 cells (data not shown). These observations indicate that CrFK cells may possess fewer IFN receptors than fcwf-4 cells and therefore be less sensitive against antiviral activities, allowing higher replication of IFN-sensitive viruses, as was previously suggested by Mochizuki et al. (1994).

In summary, it was clearly demonstrated that protein 7a is a key player in the circumvention of the antiviral defence of the host. Moreover, cooperation between protein 7a
and ORF3-encoded proteins appears to be essential to completely abolish IFN-α-mediated restriction of viral replication. As the exact mechanism of counteraction remains unclear, this leaves abundant scope for further unravelling of the molecular mechanism employed by 7a to antagonize the IFN-induced antiviral response. This will aid in defining innovative antiviral reagents that work by preventing FIPV from blocking specific cellular activities, supporting the defeat of this disease.

**METHODS**

**Viruses and cells.** CrFK cells were used to obtain third passages of type II FIPV strain 79-1146. FIPV strain 79-1146 was kindly provided by Dr P. J. M. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands).

Fcwf-4 cells were used to obtain fifth passages of FIPV-A7. This virus is a deletion mutant from type II FIPV strain 79-1146, kindly provided by Dr P. J. M. Rottier. It has been deleted in ORF7ab, using reverse genetics (Haijema et al., 2004).

The 293FT cell line, which is a fast-growing, highly transfectable clonal isolate derived from human embryonic kidney cells transformed with the simian virus 40 large T antigen, was used for production of lentiviruses containing the 7a or 7b gene.

**Generation of fcwf-4 cells stably expressing FIPV 7a and 7b protein.** The genes for FIPV 79-1146 7a and 7b proteins, with stop codons mutated into tyrosine and serine, respectively, were synthesized by GenScript. Genes were cloned into a lentivector pTRIP-CMV-GFP-WPRE. It was verified that the accessory protein 7a and 7b genes were in frame with the reporter GFP gene, resulting in the expression of 7a and 7b proteins with a GFP tag at their C terminus.

To produce a lentivirus, three plasmids were transfected into a packaging cell line, 293FT, by the use of a calcium phosphate transfection kit (Invitrogen). One plasmid, generally referred to as packaging plasmid, encodes the virion proteins, such as the capsid (gag gene) and the polymerase (pol gene). The second plasmid is the envelope vector containing the VSV G protein. The third plasmid is the pTRIP-7a7b-GFP transfer vector, which contains the genetic material (7a/7b-GFP) to be delivered by the vector. After 3 days, lentivirus supernatant was harvested and was used to transduce fcwf-4 cells. 7a- or 7b-expressing fcwf-4 (fcwf-7a or fcwf-7b) cells were selected by FACS using a FACSArray cell sorter (BD Biosciences) to select for GFP expression and further cultivated. Expression and localization of the 7a/7b–GFP fusion proteins was verified by fluorescence microscopy (Leica Microsystems DMRBE) and Western blotting.

**Antibodies for fluorescence staining.** Primary calnexin antibody C-20 was purchased from Cell Signaling and used at a 1/50 dilution. Primary mouse anti-Golgi 58 kDa protein/formiminotransferase cyclo-deaminase mAb was purchased from Sigma and used at a 1/100 dilution. Secondary Alexa Fluor 594-conjugated rabbit anti-goat antibody was used at a 1/500 dilution and Texas red-conjugated rabbit anti-mouse antibody was used at a 1/100 dilution; both were purchased from Molecular Probes. Membrane staining was performed by incubation of the cells with 2 mM biotin (Thermo Scientific), followed by streptavidin–Texas red (Molecular Probes) staining (1/100). Cell nuclei were stained with Hoechst 33342 (Molecular Probes).

**SDS-PAGE and Western blotting.** Stably transduced cells were scraped into medium on ice, washed in PBS buffer and lysed in lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 % SDS, 1 % NP-40, 1 mM NaF, 1 mM Na3VO4, 1 mM PMSF, Protease Inhibitor Cocktail) for 1 h at 4 °C. Cell lysates were sonicated on a 12 % polyacrylamide gel by SDS-PAGE and then transferred to a Hybond-P PVDF membrane. After blotting, the membranes were blocked in 5 % non-fat dried milk in 0.1 % PBS/Tween 20 overnight at 4 °C. Following 1 h incubation with primary Antibody recombination rabbit GFP mAb (Molecular Probes) and 1 h incubation with secondary goat anti-rabbit HRP-conjugated antibody (Dako Cytomation), blots were developed by enhanced chemiluminescence (ECL Prime; GE Healthcare) and analysed with a ChemiDoc MP Imaging System (Bio-Rad). All incubation steps were done in blocking buffer.

**IFN-α detection bioassay.** Sera from four FIP cats (confirmed by post-mortem examination), three FECV-negative cats and three FECV-positive cats were used for the detection of in vivo IFN production. In vitro samples were supernatants of fcwf-4, fcwf-7a, fcwf-7b and fcwf-GFP cells infected with FIPV-wt or FIPV-A7 (m.o.i. of 0.02). Twofold serial dilutions were made from the samples, and virus in the samples was inactivated by UV treatment (CL-1000 UV Cross-linker, UVP). IFN-α bioactivity was measured by an fcwf-4-cellassed bioassay, fcwf-4 cells (3 × 10^4 cells per well in 100 μl) were incubated overnight at 37 °C in twofold dilutions of samples or recombinant feline IFN-α as the standard (two repeats per sample). Subsequently, VSV was added to the wells, and the cells were incubated for 2 days. It is known that VSV is extremely sensitive to the antiviral actions of IFN-α (Belkowski & Sen, 1987). Viability staining of the cells was done by incubation with 0.1 % neutral red, and after washing the absorbance was read using a spectrophotometer (Repetto et al., 2008). The dilution mediating 50 % protection was defined as 1 U IFN-α per 50 μl.

**Infection.** Monolayers of cells (fcwf, fcwf-7a and fcwf-7b) were treated with increasing amounts (0–10^10 U ml^-1) of recombinant FIFN-α (R&D Systems) for 24 h prior to infection. Cells were then inoculated with FIPV-wt strain 79-1146, FIPV deletion mutants FIPV-A7 and FIPV-D3Δ7, or VSV at an m.o.i. of 0.02. IFN-α remained present during infection. FIPV-infected cells were fixed for staining, and virus produced was harvested for titration at 18 h post-inoculation (p.i.). Supernatant of VSV-infected cells was harvested for virus titration at 18 h p.i.

**Detection of viral N protein expression in FIPV-infected cells.** Fcwf-4 cells were trypsinized and fixed with 4 % paraformaldehyde. FIPV N protein expression was visualized with mouse IgG1 mAb 10A12 (produced and characterized in house) followed by FITC- or Alexa Fluor 647-labelled goat anti-mouse IgG secondary antibodies (Molecular Probes). The specificity of the antibody binding was assessed by substituting the primary anti-N antibody with an isotype-matched irrelevant antibody. The percentage of infected cells for every virus was determined using FACS Diva software on a FACSCanto flow cytometer (BD Biosciences), and the percentage reduction in N-positive cells in IFN-treated versus untreated cells was calculated.

**Virus titration.** Total virus was obtained by scraping the cells into their supernatants. Cells and supernatant were transferred to an Eppendorf tube and subjected to one freeze–thaw cycle. Virus titres were assessed by TCID50 assay using CrFK cells. The 50 % end point was calculated according to the method of Reed & Muench (1938). The percentage reduction in virus titre in IFN-treated versus untreated cells was calculated.

**Statistical analysis.** The percentage reduction in N-positive cells and virus titres in IFN-treated versus untreated fcwf-4 cells (whether transduced with 7α–GFP, 7β–GFP or GFP, or not transduced) was calculated. Statistical analysis was performed by Mann–Whitney U test.
test with SPSS 19.0 (SPSS). Differences were considered significant if \( P \leq 0.05 \).

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REFERENCES


