3C protein of feline coronavirus inhibits viral replication independently of the autophagy pathway

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ABSTRACT

Feline coronavirus (FCoV) can cause either asymptomatic enteric infection or fatal peritonitis in cats. Although the mutation of FCoV accessory gene 3c has been suggested to be related to the occurrence of feline infectious peritonitis (FIP), how the 3C protein is involved in this phenomenon remains unknown. To investigate the role of the 3C protein, a full-length 3c gene was transiently expressed and the cytoplasmic distribution of the protein was found to be primarily in the perinuclear region. Using 3c-stable expression cells, the replication of a 3c-defective FCoV strain was titrated and a significant decrease in replication ($p < 0.05$) was observed. The mechanism underlying the decreased FIPV replication caused by the 3C protein was further investigated; neither the induction nor inhibition of autophagy rescued the viral replication. Taken together, our data suggest that the 3C protein might have a virulence-suppressing effect in FCoV-infected cats. Deletion of the 3c gene could therefore cause more efficient viral replication, which leads to a fatal infection.

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1. Introduction

Feline coronavirus (FCoV) is an enveloped virus belonging to genus *Alphacoronavirus*, family *Coronaviridae*, order *Nidovirales*. Other closely related group 1a CoV members include canine coronavirus, transmissible gastroenteritis virus and porcine respiratory coronavirus. The genome size of FCoV is approximately 28.9 kb and encodes 1 replicase gene, 4 structural genes, the S, E, M and N genes and 5 group-specific accessory genes, including 3abc and 7ab (Lai et al., 2007).

FCoV is an important pathogen in domestic and non-domestic cats and can be further categorized into 2 biotypes: FECV, which accounts for enteric infections, and FIPV, which leads to the highly lethal disease feline infectious peritonitis (FIP) (Pedersen, 2009). In addition to variation in the *in vivo* pathogenicity, the two viruses replicate differently *in vitro* as well (Pedersen, 2009). Infection of feline peritoneal macrophages by FIPV results in more infected cells, a higher titer of viral progeny, more efficient spreading to other susceptible cells and greater sustained infection than FECV (Stoddart and Scott, 1989). Similar findings have also been demonstrated in feline peripheral blood monocytes (fPBM) (Dewerchin et al., 2005).

Several genes have been suggested to be related to the difference in virulence, including S (Chang et al., 2012; Regan et al., 2008; Rottier et al., 2005), 3abc (Balint et al., 2012; Chang et al., 2010; Pedersen et al., 2012; Vennema et al., 1998) and 7ab (Herrewegh et al., 1995; Kennedy et al., 2001). Recently, the integrity of the 3c gene has been found to be correlated with the outcome of infection (Balint et al., 2012; Chang et al., 2010; Pedersen et al., 2012; Vennema et al., 1998). Deletion of the 3c gene has been observed in many FIPV isolates (Vennema et al., 1998) and naturally FCoV-infected cats (Chang et al., 2010; Pedersen et al., 2012). In cats that are asymptomatically infected with FCoV, nearly all of the 3c genes were intact. In contrast, most of the 3c genes of FCoVs from FIP cats harbored point mutations and/or insertion/deletion mutations that led to a truncation of the 3C protein. The 3C protein has been suggested to be related to intestinal tropism (Chang et al., 2010; Pedersen et al., 2012) and the truncated version of open reading frame (ORF) 3abc has been demonstrated to play a role in the efficient macrophage/monocyte tropism of type II FIPV (Balint et al., 2012).

Autophagy is a degradation pathway that is important for the maintenance of many physiological functions and required for the growth and survival of normal cells. Recently, autophagy has also been identified as playing a role in the innate immune system, along with RNA interference and Toll-like receptors, and has been shown to be involved in either the clearance or enhancement of viral infections (Dreux and Chisari, 2010).

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Neither direct linkage between the occurrence of FIP and the truncated 3C protein, nor the function of the intact 3C protein has ever been demonstrated. In this study, the correlation of 3c gene mutation with FIP was first confirmed by analyzing local isolates. The role of the intact 3C protein in the replication of FCoV was further investigated and its possible mechanism was discussed.

2. Materials and methods

2.1. Specimens

From 2008 to 2010, a total of 93 rectal swabs or fecal samples from the quarantine ward of National Taiwan University Animal Hospital, animal hospitals in the Taipei area, or personally owned cats were collected for the surveillance of the 3c gene of FCoV from asymptomatically infected cats. For FCoV from FIP cats, organs or body effusions collected from 32 pathologically confirmed FIP cats from 2006 to 2012 were surveyed.

2.2. Amplification of the 3c gene

The amplification of the 3c gene from healthy and FIP cats was performed by reverse transcription-nested polymerase chain reactions (RT-nPCRs). Briefly, the total RNA of different specimens were extracted and reverse transcribed with a template-specific primer for the M gene (M154-R). cDNAs were then amplified with primers for the 3′ end of S gene and 5′ end of M gene (S4198-F and M154-R), followed by a second round of amplification with different combinations of primers specific to either the 3a or 3b gene and E gene. The sequence and position corresponding to the sequence of FCoV/NTU156/P/2007 (NTU156) (GenBank accession No.: GQ152141) are listed (Table 2). The products of the RT-nPCRs were electrophoresed and the amplicons were excised from the agarose gel, purified with a gel extraction kit (Geneaid, Taipei, Taiwan) and sequenced (Mission Biotech, Taipei, Taiwan).

2.3. Cells and virus

Felis catus whole fetus-4 (Fcwf-4) cells (kindly provided by Professor Peter J. M. Rottier, Utrecht University) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. FCoV NTU156 is an FCoV strain recently isolated from pleural effusion of a kitten by the co-cultivation method (Lin et al., 2009). NTU156 harbors a 434-nucleotide deletion that leads to a 67 amino acid-truncated 3C protein. The complete genome sequence of strain NTU156 was deposited in GenBank under the access number GQ152141. All of the viruses used in this study for the assessment of the effect of 3C protein expression came from a stock passaged 10 times.

2.4. Construction of a plasmid for the expression of full-length 3C protein

The full-length 3c gene was amplified from the rectal swab of a naturally FCoV-infected cat with enteric infections (GenBank accession No.: DQ160294). Subsequently, the amplified products were cloned into mammalian expression vectors, pCi-neo (Promega, Madison, USA), and named pCi-3cHis.

2.5. Establishment and confirmation of the 3C protein-expressing cells

Fcwf-4 cells were transfected with either pCi-neo or pCi-3cHis using Lipofectamine™ reagent (Invitrogen, Carlsbad, USA) and se-

lected with G418 (Gibco, Grand Island, USA) for the generation of stable expression cells. Mixed populations of G418-resistant cells generated from the transfectant of pCi-neo and pCi-3cHis will be designated as Fcwf-Ci and Fcwf-3cHis cells, respectively. The cells were than fixed and stained with mouse anti-6 histidine mAb (Invitrogen, Carlsbad, USA) as the primary antibody and FITC-conjugated goat anti-mouse IgG antibody (Leinco Technologies, St. Louis, USA) as the secondary antibody. The stained cells were observed under an inverted fluorescence microscope.

2.6. Effect of the expression of the 3c gene on the replication of FCoV

Fcwf-Ci and Fcwf-3cHis cells were infected with FCoV NTU156 at an MOI of 0.1. After 1 h of adsorption, the inocula were removed, cells were washed 3 times with PBS and the media was replaced with fresh DMEM containing 2% FBS. The culture supernatants were harvested at 6, 12 and 24 h after infection and titred by a plaque assay.

2.7. Involvement of autophagy in the effect of the 3C protein

To confirm the regulation of the autophagy activity by the treatment of chemical autophagy regulators, Fcwf-3cHis cells were treated with FBS-free DMEM containing an autophagy inhibitor, 3-methyladenine (3-MA) at 100 μM (Sigma, St. Louis, USA), or DMEM containing 10% FBS and rapamycin, an autophagy inducer, at 100 nM. After cultivation for 2 h, cells were washed with cold PBS for three times and lysed with RIPA buffer containing 20 μM Pefabloc® SC (Merck, Darmstadt, German), boiled with 4X SDS–PAGE loading buffer for 10 min and stock at −80 °C before use. For monitoring the autophagy activity by Western blotting, the cell lysates containing 15 μg total protein were resolved in 15% SDS–PAGE and transferred to the polyvinylidene difluoride membrane. LC3-II was first probed by the rabbit anti-LC3 antibody (Novus, Littleton, USA) and followed by HRP-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, USA). For the detection of β-actin, mouse anti-β-actin antibody (Novus, Littleton, USA) and HRP-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, USA) was used. The signals were captured with imaging system and semi-quantified by analyzing the signal density using ImageJ 1.46 (U.S. National Institutes of Health, Bethesda, USA) (Schneider et al., 2012).

For the evaluation of the involvement of autophagy in the effect of the 3C protein, similarly, Fcwf-3cHis cells were treated with either 3-MA or rapamycin and cultured for 2 h. Subsequently, the culture supernatant was removed and the cells were infected with FCoV NTU156 at an MOI of 0.1. Subsequently, the inoculum was removed and replaced with fresh DMEM containing 2% FBS. The culture supernatant was then harvested after 12 h of cultivation and the viral titer was determined.

2.8. Statistical analysis

To test the correlation between the mutation of the 3c gene and the occurrence of FIP, Fisher's exact test was performed and p values <0.05 were considered to be significantly correlated. The data from the cells transfected with different plasmids are presented as the mean ± standard deviation. Student's t-test was used for comparisons between 3c-expressing and non-expressing groups. p values <0.05 were considered to be statistically significant.

3. Results

3.1. Integrity of the 3c gene of FCoV from FIP and asymptomatic cats

To confirm the correlation between the integrity of the 3c gene and occurrence of FIP in our local naturally infected cats, 93 rectal

swabs or fecal samples were analyzed, of which 43 (46.2%) were positive for FCoV infection. The region that included the full-length 3c gene was successfully amplified from 21 of the 43 positive samples. 3c genes from enterically infected cats were mostly intact, except for one cat (Fig. 1A; Table 1). The results from the genetic analysis showed that there was a 3-nucleotide deletion at position 58–60 that leads to a one amino acid deletion at position 20 of the putative 3C protein. These results showed that there was a low mutation rate of the 3c gene in FCoVs from the non-FIP cats.

Of the 32 FIP cats, the region that includes the 3c gene was successfully amplified from 20 cases and 25 sequences were generated. A mutation in the 3c gene was observed in all but four of the cats (Fig. 1B; Table 1). Of the mutations, 11 mutations were deletion events and the remaining mutations were nonsense mutations. All of the mutations led to a truncation of the putative 3C protein.

3.2. Expression of the 3C protein

As the 3c gene is an accessory gene in FCoV and its expression in cells has never been reported, the 3C protein expression in Fcwf-3cHis cells was investigated by immunofluorescent assay (IFA). Around 50% Fcwf-3cHis cells were found positive (data not shown)

and the 3C protein was distributed primarily in the perinuclear region (Fig. 2). Since control plasmid pCi-neo does not include a 6 histidine tag, no fluorescent signal was detected on Fcwf-Ci cells as expected. Despite the successful observation of the 3C protein using IFA, we failed to detect the protein by Western blotting (data not shown).

3.3. Changes in viral replication in 3c-expressing cells

To determine the influence of the 3C protein in FCoV replication, a 3c-deleted strain of FIPV (NTU156) was inoculated onto 3c-expressing cells at an MOI of 0.1 and its growth was evaluated. The replication of NTU156 showed a delay in Fcwf-3cHis cells when compared with the replication in Fcwf-Ci cells. At 6 h post-infection, the extracellular virus could be readily detected in the culture supernatant of Fcwf-Ci cells. In contrast, no extracellular virus could be detected at this time point in Fcwf-3cHis cells (Fig. 3). The free viral progeny was only detected after 12 h post-infection and at a significantly lower viral titer (37.5%) in the presence of the 3C protein (Fig. 3). The slower and decreased viral titer in the 3C protein-expressing cells indicated that the 3C protein is an antiviral factor during replications.
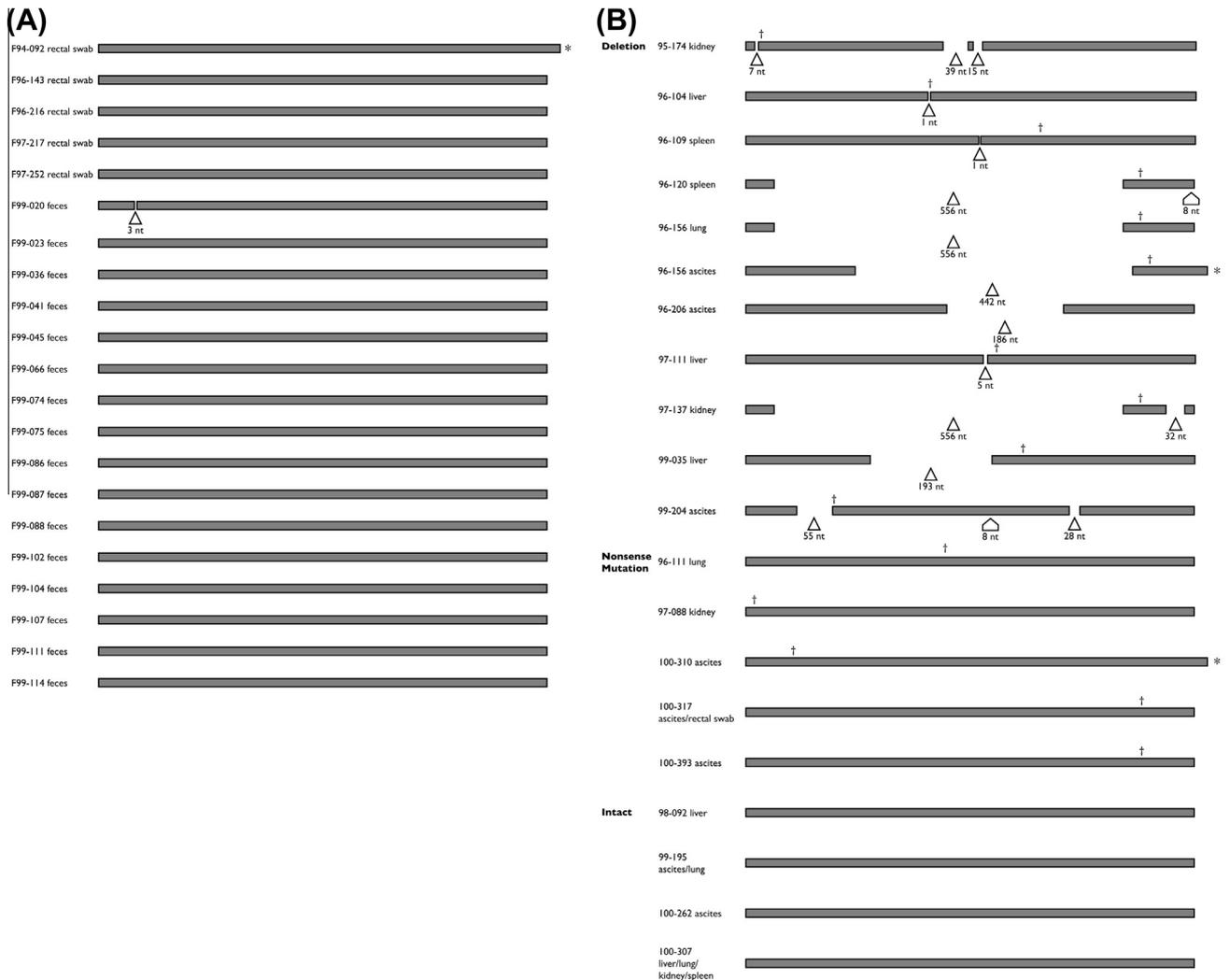

Fig. 1. The 3c gene of FCoV from different manifestations of infection. (A) Healthy cats. (B) FIP cats. The 3c gene from FIP cats showed a high degree of mutation and was strongly correlated with the development of FIP. †: Premature stop codon. Triangle: deletion. Pentagon: insertion. *Type II FCoV harboring a CCoV-like 3c gene.

Table 1
Basic information about the healthy and FIP cats involved in this study.

Category	Cat no.	Age	Gender	Breed[a]	Genotype[b]	Type of mutation	Truncation (a.a.)[c]
Healthy							
	94-092	2.5 months	F	DSH	II	–[d]	0
	97-216	2 years	M	DSH	I	–	0
	97-217	2 years	M	DSH	I	–	0
	97-252	5 years	F	DSH	I	–	0
	99-020	4 years	M	DSH	I	–	0
	99-022	2 years	F	DSH	I	–	0
	99-037	8 years	M	DSH	I	Deletion	1
	99-041	4 months	F	DSH	I	–	0
	99-045	9 years	F	DSH	I	–	0
	99-047	5 years	M	DSH	I	–	0
	99-074	7 years	F	ASH	I	–	0
	99-075	3 years	M	Persian	I	–	0
	99-086	2 years	M	DSH	I	–	0
	99-087	5 years	F	Persian	I	–	0
	99-088	5 years	M	DSH	I	–	0
	99-102	4 years	M	ASH	I	–	0
	99-104	3 years	M	Chinchilla	I	–	0
	99-107	2 years	F	DSH	I	–	0
	99-111	4 years	M	DSH	I	–	0
	99-114	3 years	M	Persian	I	–	0
	99-253	12 years	F	ASH	I	–	0
FIP							
	95-174	5 months	F	DSH	I	Deletion/PT[e]	230
	96-104	3 months	M	Scottish fold	I	Deletion/PT	140
	96-109	1 year	F	Chinchilla	I	Deletion/PT	82
	96-111	4 months	F	DSH	I	Nonsense mutation	132
	96-120	1.5 years	M	Persian	I	Deletion/PT	214
	96-156	4 months	M	DSH	I/II	Deletion/PT	214/178
	96-206	3 years	F	DSH	I	Deletion	62
	97-088	1 year	F	DSH	I	Nonsense mutation	234
	97-111	1.5 years	M	ASH	I	Deletion/PT	105
	97-137	2 years	F	DSH	I	Deletion/PT	214
	98-092	3 months	M	DSH	I	–	0
	99-035	2 years	M	DSH	I	Deletion/PT	161
	99-195	3 months	M	DSH	I	–	0
	99-204			ASH	I	Deletion/PT	159
	100-262	4 years	M	DSH	I	–	0
	100-307	3.5 years		DSH	I	–	0
	100-310		F	DSF	II	Nonsense mutation	220
	100-317	1.5 years		DSH	I	Nonsense mutation	28
	100-393	2 years	M	DSH	I	Nonsense mutation	28

[a] DSH: domestic short hair; ASH: American short hair.
[b] Based on the sequence similarity of 3c gene to FCoV (I) or CCoV (II).
[c] Numbers of the amino acid of the 3C protein shortened comparing to the intact 3C protein of FCoV (238 amino acids) or CCoV (245 amino acids) for genotypes I and II, respectively.
[d] Non-mutations were found.
[e] Premature termination.

3.4. The 3C protein-mediated inhibitory effect on FCoV replication is not mediated by autophagy

Recently, autophagy has been shown to play an important role in the regulation of many viruses. To further investigate whether the decreased FIPV replication led by the 3C protein is mediated by autophagy, Fcwf-3cHis cells were treated with different chemical regulators of autophagy, including an autophagy inhibitor, 3-MA and rapamycin, an autophagy inducer, and the changes in autophagy activity and viral replication were evaluated. Despite of the increased or deceased autophagy activity triggered by the addition of rapamycin (Fig. 4A) or 3-MA (Fig. 4B) on the 3C pro-

Table 2
Primers used in this study to amplify the regions containing the 3c gene of FCoV. Combination of different set of primers were used to achieve successful amplification.

Primer	Sequence (5′–3′)	Position[a]	Orientation	Reference
1st PCR				
S4198-F	AAT GGC CTT GGT ATG TGT GG	29,897–24,916	Sense	This study
M154-R	ACA GAC CAG CTG AAG TTC AGT T	26,266–26,244	Antisense	This study
2nd PCR				
3a151-F	GTG TGT ATA GGT TTT GGT G	24,921–24,939	Sense	This study
E68-R	AAT ATC AAT ATA ATT ATC TGC TGG A	25,917–25,893	Antisense	This study
3b169-F	CAA GTA CTA TAA AAC GTA GAA GMA G	25,478–25,502	Sense	Chang et al. (2010)
E50-R	CAG GAG CCA GAA GAA GAC ACT AA	25,897–25,875	Antisense	Chang et al. (2010)

[a] The position of the target sequence referred to the genome of FCoV isolate Black (GenBank accession No.: EU186072).
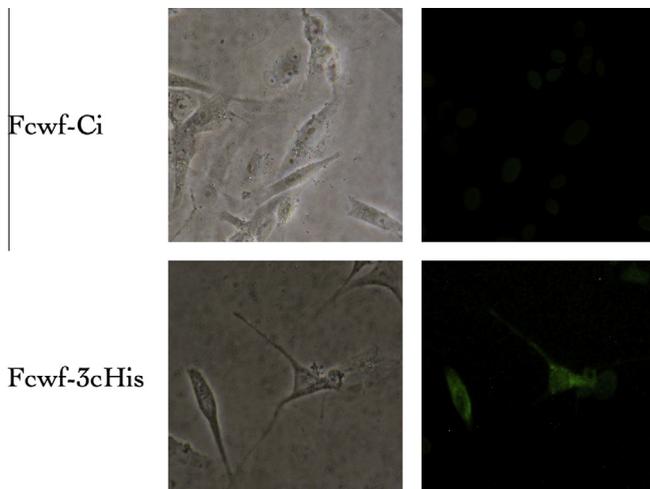
Fig. 2. Expression of the 3c gene. Fcwf-3cHis or Fcwf-Ci cells were stained with an anti-6 histidine tag mAb and observed under an inverted fluorescence microscope.
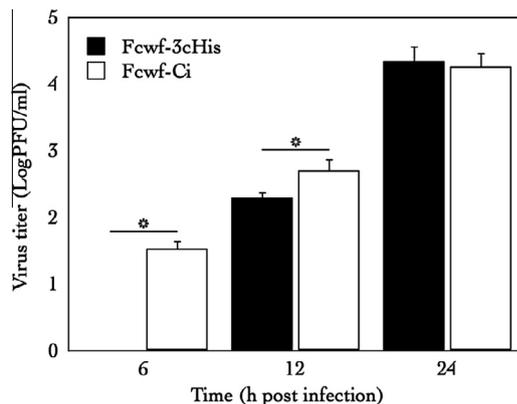

Fig. 3. Effect of the expression of the 3C protein on the replication of FCoV. Fcwf-3cHis or Fcwf-4 cells were infected with a 3c-defective FCoV isolate. The culture supernatants were used to titer the infectious viral progeny by a plaque assay. The viral titer is shown as log PFU/ml. *$p < 0.05$ vs. control group.

tein-expressing cells, viral replication remained the same (Fig. 4C). These results indicated that the inhibition of FIPV replication by the 3C protein is not through the regulation of autophagy.

4. Discussion

The correlation between the integrity of the 3c gene of FCoV and disease manifestation has recently begun to be studied intensively. Because the number of animals analyzed in each study was relatively limited to gain a clearer insight into this correlation, we summarized the results obtained in this study together with other results (Chang et al., 2010; Pedersen et al., 2009, 2012; Vennema et al., 1998). Full-length 3c genes were analyzed from a total of 166 cats, 76 of which were asymptomatically infected and 90 of which had FIP (Table 3). The 3c genes of FCoV from clinical healthy cats were mostly intact (97.4%), except for two isolates (2.6%) and the mutated 3c gene from these two asymptomatic cats resulted in only one amino acid difference of either insertion (Pedersen et al., 2012) or deletion (this study). In contrast, in isolates from the 90 FIP cats, 67 (72.2%) were found to be infected with at least one FCoV strain that harbored a mutated 3c gene (Table 3). These

observations indicated that the mutation of the 3c gene is strongly correlated with the occurrence of FIP ($p < 0.001$).

The integrity of 3c-like genes and disease manifestation has been studied in other group 1 CoVs. In ferret coronavirus, which is similar to FCoV, the mutation of a 3c-like gene is correlated with systemic infection (FIP-like disease manifestation) (Wise et al., 2010). However, in transmissible gastroenteritis virus (TGEV) in pigs and CCoV in dogs, mutations in this gene have been suggested to be related to attenuation of the viruses (Decaro et al., 2007; Horsburgh et al., 1992; Sanchez-Morgado et al., 2004; Zhang et al., 2007). To the best of our knowledge, there is only one study involving the characterization of a 3C-related protein. In TGEV, this protein has been demonstrated to be a membrane-associated glycoprotein that is primarily distributed in the ER/Golgi (O'Connor and Brian, 1999). In this study, the pattern of the distribution of the 3C protein is similar to that of the 3c-related protein in TGEV-infected cells. We failed to detect the 3C protein through Western blotting, a similar result to that reported for the 3C-like protein of TGEV. Low abundance or fast degradation could be the reason for this result for both proteins (O'Connor and Brian, 1999).

Due to the lack of detection of a 3c-mutated FCoV in the feces from FIP cats, the 3c gene has been proposed to play a crucial role in the intestinal replication of FCoV (Chang et al., 2010; Pedersen et al., 2012). It is noteworthy that in this study, one FIP cat infected with a 3c-mutated FCoV also shed a virus harboring the same nonsense mutation through its intestinal tract (Fig. 2; cat 100-317). Although there is not sufficient evidence to confirm whether the virus replicated in the intestine, this possibility cannot be excluded.

We demonstrated in this study that the 3C protein could suppress the growth of FCoV (Fig. 3). A similar finding has been reported recently using a recombinant FIPV (rFIPV) strain DF-2 that harbored a truncated ORF3abc. The replication of rFIPV strain DF-2 with intact ORF3abc was lower in the Fcwf-4 cells by approximately 0.5 log PFU/ml and was significantly decreased in feline monocytes when compared to rFIPV DF-2 with truncated ORF3abc (Balint et al., 2012). In this study, using exogenous expression of the 3C protein, we were able to narrow down this decreased viral replication to one gene. These findings strongly indicated that the 3C protein could be a virulence-suppressing factor during FCoV infection and might play an important role in the pathogenesis of FIP.

Although a significant decrease of viral titer was observed, this inhibitory effect can only be detected in the early time points of the virus infection, i.e. 6 and 12 h post-infection. The possible reason for this transient alteration could be due to the heterogeneity of the cells used in this study. Since only 50% G418-resistant cells were expressing the 3C protein and the virus strain NTU156 we used to monitor the replication was a relatively fast growing one (6 h/replication cycle) (Lin et al., 2009), the viruses produced from cells without the 3C protein expression may hence masked the effect at the later time point.

Autophagy is a catabolic process involved in the procession of damaged organelles, intracellular protein aggregates, and the turnover of long-lived proteins and has been shown to be important for cell development and survival during nutrient deprivation. Other than its role in physiology, autophagy has also been shown to play a role in the regulation of viral replication. Many viral proteins have been determined to be detected by autophagy machinery, and viral replication has been shown to be decreased due to the degradation of viral proteins by autophagy. Hence, some viruses have evolved mechanisms that interfere with autophagy pathways to prevent the clearance of the infection (Dreux and Chisari, 2010). In contrast with this role in host defenses, some viruses have been found to utilize autophagy machinery as a site for replication, and the activation of this pathway thus facilitates viral replication. Due
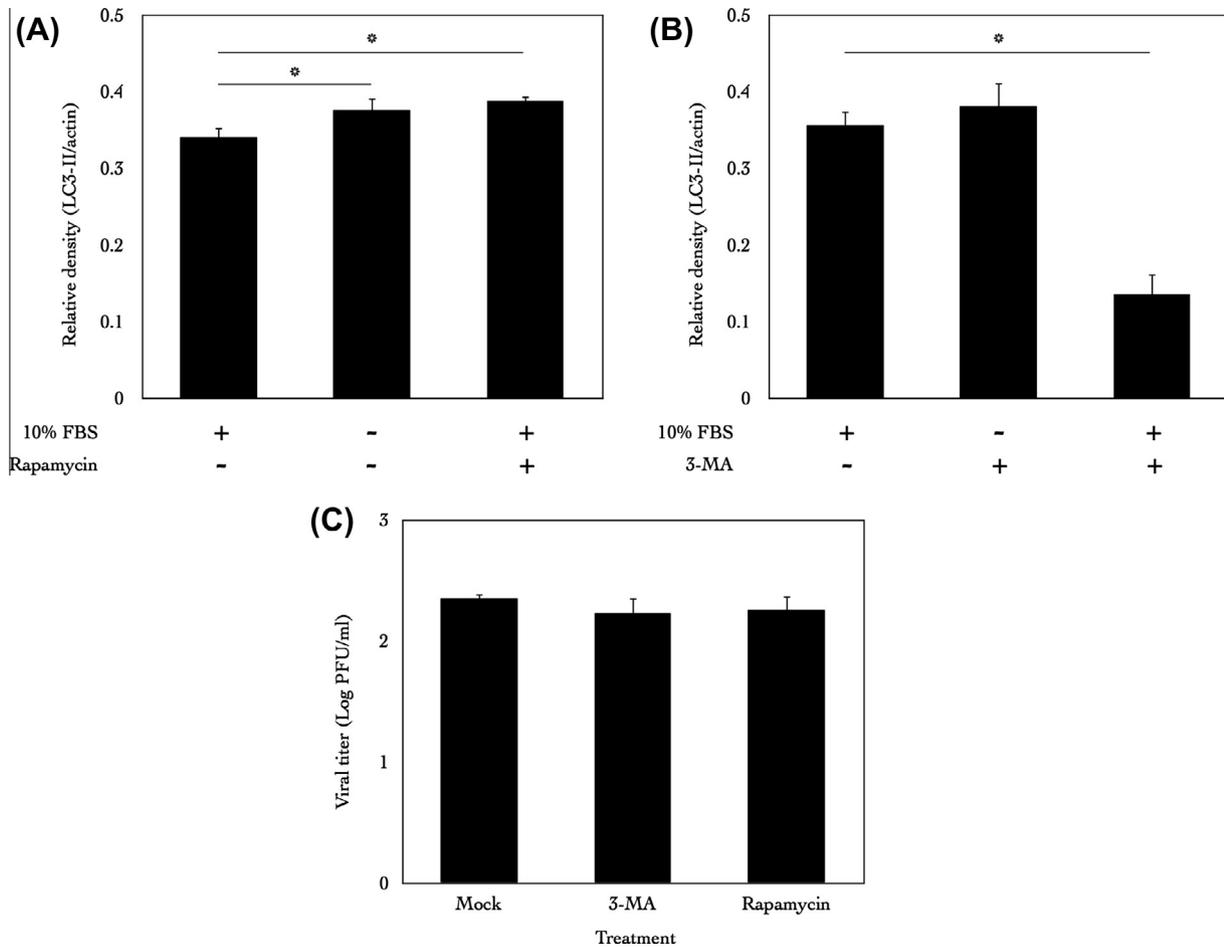
Fig. 4. Autophagy activity in Fcwf-3cHis cells and its effect on the replication of FCoV. Fcwf-3cHis cells were treated with different autophagy regulators, rapamycin (A) and 3-MA (B). Cells maintained in the DMEM with or without 10% FBS were used as negative or starvation control. Autophagy activity was monitored by Western blotting and semi-quantified by measuring the signal density of LC3-II. (C) Fcwf-3cHis cells were treated with different autophagy regulators and infected with a 3c-defective FCoV isolate. The production of viral progeny were titred after 12 h post-infection. The viral titer is shown as log PFU/ml. *$p < 0.05$ between indicated groups.

Table 3
Studies concerning the integrity of the 3c gene in different disease manifestation of FCoV around the world.

Studies	Disease manifestation				Reference
	FIP		Healthy		
	Intact	Mutated	Intact	Mutated	
1	0	2	1	0	Vennema et al. (1998)
2	0	8			Pedersen et al. (2009)
3	8	20	27	0	Chang et al. (2010)
4	13	19	26	1	Pedersen et al. (2012)
5	4	16	20	1	This study
Total number (%)[*]	25 (27.8)	65 (72.2)	74 (97.4)	2 (2.6)	

[*] Within the group.

to the importance of the autophagy pathway in innate viral defenses, the involvement of the 3C protein with autophagy was determined. However, treatment with both an autophagy inhibitor and autophagy inducer did not significantly rescue viral replication in 3c-expressing cells (Fig. 4B). This indicates that the inhibitory effect of the 3C protein is mediated by pathway(s) other than autophagy.

Viral proteins that can modulate the host response to infection and decrease their virulence have recently been found in other viruses, e.g., the PA-X protein, which is encoded by segment 3 of influenza A virus (Jagger et al., 2012), and ORF 7 protein of TGEV (Cruz et al., 2011). An intact 3C protein may play a role in similar pathways and maintain the symbiosis of virus and host. Addition-

ally, various cellular pathways have been shown to be crucial in the life cycle of different CoVs, including endosomal proteolysis (Qiu et al., 2006; Regan et al., 2008), the mitogen-activated protein kinase signal transduction pathway (Cai et al., 2007), GBF1-mediated ARF1 activation (Verheije et al., 2008), LC3-1-positive ESEMsome (Reggiori et al., 2010) and the ubiquitin–proteasome system (Raaben et al., 2010). It is also possible that the 3C protein interferes with these pathways and hinders viral replication. Identification of the underlying mechanism(s) of 3c might elucidate the pathogenesis of FCoV infection and occurrence of FIP. Knowing the pathways or machineries that the 3C protein interacts with in host cells will help us determine effective antiviral agents to block the viral life cycle and clear FCoV infection.

Acknowledgement

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