Final Report

Introduction

Feline coronaviruses (FeCoVs) are present in two different pathotypes in the field. The feline enteric coronavirus (FECV) is more common and it causes mild or unapparent enteritis, while feline infectious peritonitis virus (FIPV) is responsible for a severe systemic granulomatous disease. FECV replication is mostly confined to the intestinal tract while FIPV replicates in monocytes and macrophages. In the case of FECV/FIPV the shift of *in vivo* tissue tropism correlates with virulence and the shifting of the focus of viral replication from the intestinal tract toward macrophages and monocytes goes hand in hand with deletions in the accessory region 3 and 7. Scientific data of others and our own suggest that some ORFs (especially 3c and 7a) from these regions directly influence pathogenecity of the FeCoVs. Though there is an accumulation of direct and indirect scientific evidence that accessory proteins translated from these regions might play important roles in sustaining and restricting viral replication in the intestinal tract of the host, yet to date, our knowledge about their exact function and even about their physical properties (including their precise number and size) is still very limited. Our main objective is to uncover the molecular mechanisms behind the operation of region 3 and 7 in feline coronaviruses and to clarify their role in the sustainment of FECV's enterotropism, and in the development of systemic infection by FIPV.

The goal of the project

Our aim was to study the transcription and the translation of region 3 and 7 of the FECV/FIPV virus pair *in vitro*, as well as the localization and cellular interaction of the translated proteins in different tissues.

Investigation of the number of mRNAs produced from regions 3 and 7 of FeCoVs

The core sequences, the indicators of the potential mRNAs, show surprising heterogeneity in the region 3 of different FeCoVs. Serotype II FIPVs contain three core sequences, one for each ORF. Most of the serotype I viruses have only one core sequence at the 5'end of 3a ORF which suggests that the region's ORFs are translated from one mRNA. Region 7 of the FeCoVs has also one core sequence at the 5'end of 7a which indicates one mRNA for the translation of 7a and 7b ORFs.

We have sequenced the 3 abc region of seven new Type I FECV isolates and six FIPV isolates. As expected, the majority of the new isolates contained one core sequence (CUAAAC). To generate FECV FIPV transcription map of regions 3 and 7 in two different isolates we used RT-PCR rather than Northern blot, because it is more sensitive and it facilitates the detection of low-level synthesized mRNAs.

In FeCOVs all mRNAs start with the leader sequence, therefore we used a leader specific primer as forward primer, and region specific primers from around the 3' end of the ORFs (three primers for region 3 to 3a, 3b and 3c ORFs and two primers for region 7 to 7a and 7b ORFs) to detect the different mRNA species. In each case we detected only one major band. Sequencing of the major bands revealed that the leader sequence in both regions is in fact joined to the core sequence and both regions have only one messenger RNA. To exclude the presence of minor mRNA species, minor bands were also sequenced. None of the minor bands originated either from the RT PCR to region 3 or from the RT PCR to region 7 proved to be viral sequences, reconfirming the presence of only one mRNA for each region.

Expression of proteins of regions 3 and 7 of FeCoVs

Translation of eukaryotic mRNAs is usually initiated by only the first AUG codon, though exceptions, such as reinitiation, internal initiation and leaky scanning have all been documented in viruses.
We used eGFP and 3xFLAG tagging to investigate the translation and localization of the proteins coded by the ORFs of regions 3 and 7. Carboxy terminal (C-terminal) tagging of the proteins was chosen because none of the utilized protein function and signal prediction programs predicted such functional units whose interaction with the C-terminal tag could potentially interfere with the localization of proteins.
First we created 6 constructs of region 3 by PCR and cloned them into pEGFP-N1 vector (Clontech). The constructs contained one, two or all three ORFs of regions 3 and eGFP has been fused to the C-terminal of the last ORF of each construct. Plasmids were transfected into virus-sensitive cat-derived cell lines (FCWF and CrFK), then cells were fixed and monitored under fluorescent microscope for the presence of eGFP signal. The presence of a fluorescent signal could be detected from all six constructs inside the cells indicating the translation of the GFP-fused ORFs. The localization of the fusion proteins was similar to that of the GFP, though we expected that at least the 3c-GFP fusion protein would localise in some membranous compartment.
Similar GFP signal was detected from the three constructs (7a-eGFP, 7b-eGFP, 7ab-eGFP) of region 7. However, we started to focus more on the investigation of ORFs of region 3 because a paper was published detailing the localization and function of the proteins of region 7 (Dedeurwaerder et al. 2014 *J Gen Virol.* 2014 Feb;95(Pt 2):393-402.).
To characterize GFP fusion proteins, transfected cells of all six constructs were lysed, the fusion proteins were immuno-precipitated by anti eGFP antibodies, and their size was analysed by Western blot. Surprisingly, in all constructs – except in the 3a-eGFP construct –, the eGFP protein was the dominant band indicating that the eGFP translation is initiated by its own methionine and explaining the localization of the fluorescent signals in the cells. Interestingly, no band with the expected size (54 kDa) was detected in the case of three constructs (3a-3b-3c-eGFP, 3b-3c-eGFP and 3c-eGFP) expressing the 3c-eGFP protein. These findings suggested that native eGFP is translated from the fusion constructs that interferes with the detection of the viral fusion proteins and that 3c-eGFP is very weakly expressed or unstable in the transfected cells.
To eliminate the interfering effect of the translated native eGFP, the three ORFs of region 3 were cloned in the p-MeGFPN1 vector, which contains a mutation in the initiation codon of eGFP (Olasz et al., 2016), thereby eliminating native eGFP translation. In these fusion constructs translation can be initiated only from the ATGs of the cloned ORFs. This approach proved to be successful and clear, eGFP background-free signals could be detected in the transfected cells that allowed us to determine the localization of the 3a, 3b and 3c proteins.

Localization of proteins of region 3 and 7

The 3a protein does not contain any short basic stretches of amino acids or any recognisable classical nuclear localization signals (NLS). Yet, the majority of the 3a-eGFP protein is localised in the nucleus, though it can also be detected in the cytoplasm. Since eGFP alone shows somewhat similar distribution in the CRFK cells (nuclear and cytoplasmic), we also transfected a construct where the 3a protein was fused to a 3xFLAG-tag. Detection of the 3xFLAG-tag with anti-FLAG antibody verified the nuclear and the cytoplasmic localization of the 3a protein. The nuclear localization of the protein can indicate a DNA- or RNA-binding function, and in fact, prediction programs found potential DNA-binding amino acids in the 3a protein and revealed not one, but two regions between amino acids 12–20 and 46–63 that could serve as potential transcription activator domains (TADs).

In the majority of the cells 3b-eGFP protein was localised as a granular/speckled pattern in the cytoplasm strongly resembling mitochondrial localization. To gain fool-proof results, the 3b-eGFP and 3b-3xFLAG constructs were co-transfected with the pDsRed2-Mito plasmid expressing the mitochondrial human cytochrome-c oxidase protein fused to the dsRed fluorescent protein. In the co-transfected cells the green and the red fluorescent signal co-localised, verifying that the 3b protein resides in the mitochondria. Besides the mitochondrial localization, in around one-third of the transfected cells the 3b-eGFP protein was also clearly visible in the nucleolus. In a few transfected cells (1/100) very tight perinuclear accumulation or possibly nuclear membrane localization of the 3beGFP-labelled protein was observed.

The 3c protein showed very weak expression and 3c-eGFP and 3c-3xFLAG fusion proteins could be detected in only a very small fraction of the CrFK cells (less than one in a hundred). In these cells the fluorescent signal is localised in small point-like structures diffused in the cytoplasm that seem to aggregate in a larger structure near the nucleus. Interestingly, the GFP signal did not co-localise with the signal of the ER marker calreticulin, suggesting that 3c is not localised in the ER as suggested by Hsieh et al. (2013). Localization of the 3c protein was also investigated in the intermediate-to-trans Golgi, in the peroxisome and in the ERGIC by staining the transfected cat-derived cell lines with Lectin GS-II binding α- and β-*N*-acetyl-Dglucosaminyl residues anti-ABCD3 (recognising peroxisome-resident ABCD3 protein) and anti-ERGIC antibodies. The 3c protein co-localised with neither the peroxisome nor the ERGIC markers. Unfortunately, Lectin GS-II bound to neither Fcwf-4 nor CrFK cells. To gain additional data about the localization of 3c, the 3c-eGFP-expressing construct was transfected into MARC-145 cells (derived from African green monkey kidney) in which Golgi is readily stained by Lectin GS-II. Surprisingly, 3c was expressed much more strongly in MARC-145 cells than in the established cat cell lines (detected in one tenth of the cells), but it showed similar distribution and localization.

Interaction of proteins region 3 and 7 with each other and other viral proteins

To investigate the effect of other viral proteins on the localization and stability of the proteins of region 3, CrFK cells were transfected with 3a-c expressing plasmids and the cells were infected with FIPV-DF2. Viral infection had no visible effect on the localization of either 3a and 3c, and 3c protein was still expressed very poorly in CrFK cells. However, 3b could be detected much more frequently (~ 50%) in the nucleolus of infected non-syncytial cells than in that of the non-infected cells. In infected syncytial cells this difference cannot be observed. The perinuclear or nuclear membrane localization of 3b was also more frequent (1/10) in syncytial cells.

We also studied the protein interactions of the FIP 3a, 3b, 3c, 7a and 7b proteins in the CYTH yeast two-hybrid system. No interactions were found between the proteins with this method. However, the FECV 3a protein cloned into one of the bait vectors (pLexA-N expressing a DNA-binding domain) displayed strong transcriptional activator potential, investigation of the interaction of the FIPV (DF2) 3a protein (which is a C-terminally deleted version of the FECV 3a) revealed that FIPV (DF2) 3a by itself did not activate the transcription when it is fused to the LexA domain, which strongly suggests that the activator domain is missing from FIPV (DF2) 3a and it can be found on the C-terminal region of the FECV 3a protein (as predicted by TAD prediction tool).

Nuclear localization of the 3a protein, prediction of a transcription activator domain and our experiments all suggest that the protein is involved in gene regulation. Since these proteins act usually in complexes, most of them contain a domain that is responsible for protein-protein interactions. In fact, interaction was found between FIPV (DF2) 3a-LexA fusion protein and GAL4Ad-FIPV (DF2) 3a. Based on the above results it seems very probable that 3a in FECV-

infected cells it works as a homo-polimer (most probably dimer) transcription regulator, and it contains an activator domain on its C-terminal end. In certain FIPV strains, where 3a is partially or fully deleted, the transcription activator function became damaged or vanished entirely: this lost function may very well contribute to the different outcomes of the infection resulted by the two pathotypes of the feline coronaviruses.

***In vivo* study of feline coronaviruses with truncated and completed region 3**

We also collected *in vivo* data of how the completed ORF3abc alters virulence, virus shedding, viremia, viral load of organs and humoral immune response against type II FCoV.

For the experiments two viruses were used with deleted region 3: the FIPV-DF-2, the PBFIPV-DF-2 virus that was rescued from the molecular clone of FIPV DF-2, and a third one, PBFIPV-DF-2-R3i (a derivate of PBFIPV-DF-2), that was re-engineered to contain the intact ORF3abc region from the closely related canine coronavirus.

While the parent virus FIPV DF-2 induced FIP in all the infected cats, its recombinant virus PBFIPV-DF-2, differing only in seven nucleotides, proved to be surprisingly low virulent, although it caused an acute febrile episode similarly to the original FIPV DF-2. PBFIPV-DF-2 infection induced significantly lower virus neutralization titers than its parent virus, and lacked the second phase of viremia and development of the fatal course of the disease.

The differences between the biological properties of the two viruses with truncated ORF3abc were substantial, but by far less pronounced than it can be observed between the ORF3abc-deleted and ORF3abc-completed FCoVs. PBFIPV-DF-2-R3i genome was invariably absent in the blood monocytes. As a possible consequence of the absence of viremia, viral load of organs was not detected, the presence of PBFIPV-DF-2-R3i was found only in the mesenteric lymph node of half of the infected animals. The absence of replication in blood monocytes of PBFIPV-DF-2-R3i-inoculated cats coincide with previous data collected after FECV infection studies. The weak or missing seroconversion of PBFIPV-DF-2-R3i-challenged cats can be also explained by the low or absent systemic replication of the virus. Intensive fecal shedding and virus replication was detected during the whole period of the experiment and from the ileum of the sacrificed cats challenged with PBFIPV-DF-2-R3i carrying completed ORF3abc, in contrast to the other two investigated viruses with deleted region 3.

As a summery we can state that recombinant PBFIPV-DF-2-R3i with completed ORF3abc gained biological properties that differentiate between the feline enteric coronavirus (FECV) and FIPV biotypes such as intensive replication in the gut, absence of viremia and weak or no serological response.

Our studies supplied the first experimental evidence that ORF3abc contributes to the restriction of FECV replication to the intestine *in vivo*.

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