A sequence of basic residues in the porcine circovirus type 2 capsid protein is crucial for its co-expression and co-localization with the replication protein

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INTRODUCTION

To date, two types of porcine circovirus have been described, porcine circovirus types 1 and 2 (PCV1 and PCV2) (Allan et al., 1998; Tischer et al., 1982). PCV1 was originally reported as a contaminant of the PK-15 cell line and is considered pathogenic only in young porcine fetuses (Saha et al., 2011). PCV2 is associated with several clinical manifestations collectively known as porcine circovirus diseases, with post-weaning multisystemic wasting syndrome as the most significant manifestation (Segalés, 2012).

PCV2 belongs to the genus Circovirus in the family Circoviridae, and four genotypes have been described so far: PCV2a, PCV2b, PCV2c and PCV2d (Xiao et al., 2015). PCV2 is an extremely small, non-enveloped virus with an icosahedral capsid measuring only 17 nm in diameter. Its genome comprises a circular ssDNA of only 1766–1769 nt (Huang et al., 2011b), which encodes two major ORFs: ORF1 and ORF2. ORF2 is translated into the capsid protein (Cap) (Nawagitgul et al., 2000), 60 copies of which make up the icosahedral proteinaceous shell protecting the viral genome (Crowther et al., 2003). Cap is the primary immunogenic protein and has the ability to bind to the host-cell receptor (Misinzo et al., 2006). As PCV2 exploits heparin sulfate or dermatan sulfate side chains of cell-surface proteoglycans as attachment receptors, the putative heparin-binding motif 98IRKVK103 was initially thought to be a potential binding site (Misinzo et al., 2006). Recently, however, the atomic structure of PCV2 Cap revealed that this motif is located on the inside of the capsid, excluding a role in cell-surface attachment (Khayat et al., 2011). Moreover, it appeared to be part of a larger constellation of positively charged amino acids on the inner surface of the capsid, including a notable all-conserved 97RIRKVK102 sequence strongly reminiscent of the putative heparin-binding motif. The manifest conservation of this pattern suggests a well-defined purpose for this specific stretch of basic residues. The present study employed a reverse-genetics approach to clarify its function by mutational analysis.

†Both authors contributed equally to this work.
RESULTS

Mutations in the basic residues of $^{97}$RIRKV$^{102}$ decrease Cap expression and interfere with subcellular Cap localization

In order to determine the role of the basic amino acids in the conserved $^{97}$RIRKV$^{102}$ sequence, mutants M1 (R97T, R99T), M2 (K100T, K102T) and M3 (R97T, R99T, K100T, K102T) were constructed. Sequencing confirmed that all mutations were introduced correctly. PCV-negative PK-15 cells were transfected with DNA of each mutant and the parental clone, and subsequently immunostained at different time points after transfection. The numbers of PCV2-positive cells are shown in Fig. 1(a). For M1, M2 and the parental clone, Cap was detected starting from 12 h post-transfection (p.t.), and the numbers of positive cells rapidly increased to a maximum at 36 h p.t. For M3, however, no Cap-positive cells were detected. In the first 36 h p.t., the number of PCV2-positive cells was comparable between M1, M2 and the parental clone. However, by 72 h p.t., the number of positive cells had decreased markedly for these mutant viruses, suggesting a drop in viral fitness in secondary rounds of replication.

Despite similar numbers of positive cells at 36 h p.t., immunostaining showed strikingly different Cap expression patterns (Fig. 1b). The Cap staining intensity for M1 was weaker than the parental clone, and the signal for M2 was further reduced compared with M1. The M3 immunofluorescent signal was completely absent. This prompted the question of whether the introduced mutations caused conformational changes hindering antibody binding, or whether the protein itself was expressed at a lower level. Western blotting of transfected PK-15 cell lysates confirmed that there was a reduction in the amount of Cap for M1 and M2, and a complete absence of Cap for M3 (Fig. 2a). In contrast, Rep immunostaining did not display any difference between the WT and mutants, even for M3 whose Cap expression was completely abolished (Fig. 3a). Taken together, these results demonstrated that the mutants were hampered in Cap but not in Rep expression, and that Cap expression occurred independently of Rep expression.

In addition to expression alterations, a difference in subcellular distribution was observed for Cap among the different mutants and the parental clone at 36 h p.t. (Fig. 1b). Of the parental clone-transfected cells, 3.2% showed the presence of Cap in both nucleus and cytoplasm, and another 5.1% had a positive Cap signal in the nucleus only. For M1 and M2, the number of cells that showed Cap in both the nucleus and cytoplasm dropped dramatically (1.2 and 0.8%, respectively), but this decrease was completely compensated for by an increase in Cap presence in the nucleus only (8.2 and 8.8%, respectively). Cap was never observed in the cytoplasm only. This finding showed that mutations in the $^{97}$RIRKV$^{102}$ pattern resulted in Cap being shunted towards the nucleus of transfected cells, rather than maintaining a cytoplasmic stage in its life cycle.

To determine whether the decrease in Cap expression in the cells coincided with a decrease of viral capsids released from the transfected PK-15 cells, capture-ELISA kinetics of transfected cell supernatants were studied (Fig. 2b). Over a time course of 72 h, absorbance values at 450 nm ($A_{450}$) for M1 and M2 remained lower than those for the parental clone. As expected, $A_{450}$ values for M3 were negative. Moreover, these data are perfectly in line with the intracellular capsid production data, and indicated that the conserved stretch of basic residues has no role in viral egress once the virions are assembled.

Mutations in $^{97}$RIRKV$^{102}$ hamper Cap/Rep co-localization

To determine the potential co-localization between Cap and Rep, PK-15 cells were transfected at 24 h p.t. and stained with Cap-specific mAb 12E12 and Rep-specific mAb F210 D6. M1 and especially M2 displayed a striking lack of Cap/Rep co-localization (Fig. 3a). The co-localization events were quantified by CoLocolizer Pro 2.7.1 using Manders’ coefficient correlation. This analysis revealed a Cap/Rep co-localization rate of 81, 56 and 24% for the parental clone, M1 and M2, respectively. For M3, a co-localization coefficient could not be calculated as Cap was not expressed (Fig. 3b).

Mutations in the $^{97}$RIRKV$^{102}$ drastically impact viral fitness

To investigate whether the mutant capsids released into the supernatant were still infectious, we determined the viral titres of the supernatants collected at different time points after transfection (Fig. 4a). A time-dependent rise of virus titres was observed starting from 24 h p.t. for M1, M2 and the parental clone. The highest titres were found at 60 h p.t. and were $3.9 \log_{10}$ TCID$_{50}$ ml$^{-1}$ for M1, $3.3 \log_{10}$ TCID$_{50}$ ml$^{-1}$ for M2 and $5.3 \log_{10}$ TCID$_{50}$ ml$^{-1}$ for the parental clone. The titres of the mutants were lower than that of parental clone, reflecting the dramatic decrease in Cap expression observed by immunostaining, Western blotting and ELISA. M3 supernatants were always negative for PCV2.

In order to test the fitness of the progeny virus, PCV2-transfected cell supernatants were passaged on PCV-negative PK-15 cells. A gradual decrease in virus titres reaching a level of non-detection was observed from passages 2 to 7 for the mutants, while a gradual increase in titre was observed for the parental clone (Fig. 4b). PCV2 genomic sequences obtained from passages 2, 5 and 7 for parental virus, and passages 2 and 4 for M1 and passage 2 for M2 were all 100% identical to the genome in the pCR-BluntII-TOPO vector.

Taken together, this showed that Cap with mutations in the conserved basic sequence can reach the assembly stage and
Fig. 1. (a) Total number of PCV2-positive cells at different time points after transfection. Positive cells were counted in 25 randomly selected fields. (b) Immunofluorescent staining of mutants and the parental clone by anti-Cap mAb 38C1 at 36 h post-transfection. The data represent three separate experiments performed in duplicate; results are shown as means ± SD. Magnification, ×20.
properly exit the cell for secondary rounds of infection. However, the impact on protein expression is so great that overall virus fitness was seriously compromised. Without this pattern intact, the virus is doomed to disappear from the gene pool, recapitulating the selection pressure observed in vivo.

The decrease in mutant Cap expression is due exclusively to an altered amino acid sequence, which results in a decrease in Cap mRNA translation

Next, we quantified Cap mRNA using quantitative reverse transcription PCR (RT-qPCR) to see whether the decrease in Cap expression of the mutant viruses was caused by a decrease in Cap mRNA transcription. All samples derived from the transfected PK-15 cells were positive at 12 h p.t. (Fig. 5a). Notwithstanding the differences in Cap mRNA at 36 h p.t. of the mutants compared with the parental clone, the decrease was not strong enough to fully explain the drastic decrease or even absence of protein expression. At 72 h p.t., which corresponds with the next replication cycle, Cap mRNA copy number was approximately 80% lower than the parental clone, which can be explained by the reduced virus fitness of the mutants in subsequent replication cycles. This clearly showed that the dramatic reduction in Cap protein expression at 36 h p.t. was not solely due to a decrease in Cap mRNA.

In order to completely exclude the possibility that nucleotide changes in the viral DNA or Cap mRNA would interfere with translation processes inside the cell, a new synonymous mutant, M4, was constructed. M4 had four silent nucleotide changes that did not alter the amino acid sequence of Cap. We hypothesized that, if changes at the nucleotide level influenced transcription or translation, M4 and M3 fitness would be equally affected, as both have four nucleotide substitutions in exactly the same region. Interestingly, M4 displayed a very similar phenotype to the parental clone, yielding infectious, fit viruses (Fig. 5b, c). This excluded an effect caused by changes in the nucleotide sequence itself, and confirmed that the dramatic effects on virus viability were entirely due to the altered amino acids in the mutants.

The inability of mutant Cap proteins to be expressed correctly despite ample mRNA could be explained by proteasomal degradation of the newly expressed Cap proteins. However,
PK-15 cells treated with proteasome inhibitor MG132 for 2 h at 24 h p.t. did not rescue correct Cap expression (data not shown). Thus, the picture that emerges is that mutant Cap mRNA transcripts are hampered in translation, rather than Cap proteins being degraded once expressed.

**Co-transfection with a fit genotype PCV2a virus partially rescues mutant Cap translation through an unknown mechanism**

Our findings led us to believe that the conserved basic sequence was critical in getting Cap mRNA efficiently translated into Cap. If so, co-transfection of the strain 48285-based mutants (genotype PCV2b) with the PCV2a strain LG should rescue mutant Cap expression as a result of its intact 97RIRKVK102 pattern. First, we verified that mouse mAb 8G12 only reacted with PCV2a strain LG Cap and did not cross-react with PCV2b mutants. Similarly, mouse mAb 6E9 only detected strain 48285-based mutant capsids, and did not cross-react with PCV2a strain LG Cap (data not shown). By staining mutant-transfected cells with mAb 6E9, we obtained identical results as with mAb 38C1 (Fig. 6, left column).

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**Fig. 3.** (a) PK-15 cells transfected with mutants or the parental clone, immunostained with anti-Cap mAb 12E12 (red) and anti-Rep mAb F210 D6 (green) at 24 h p.t. The images are representative of three separate experiments performed in duplicate. The inset image shows details of the outlined cell. Magnification, ×200; inset, ×3000. (b) Co-localization quantification of a mean of 60 cells per sample studied from three separate experiments performed in duplicate; results are shown as means ± SD.
As expected, co-transfection with PCV2a strain LG did not have any effect on LG expression (Fig. 6, right column). Intriguingly, this procedure partially rescued mutant Cap translation, which was particularly visible in M3/LG co-transfection (Fig. 6, middle column). This highlights once more the importance of an intact 97RIRKVK102 alignment for efficient Cap translation.

**DISCUSSION**

As the smallest of all known mammal viruses with limited genomic coding capacity, porcine circoviruses are truly fascinating biological systems. Despite their reliance on proof-reading-competent host polymerases for replication, their genomes mutate exceptionally rapidly while keeping an extremely low genetic diversity within the different PCV2 genotypes (Firth et al., 2009). In other words, most nucleotide substitutions disappear immediately under intense purifying selection and, as a consequence, PCV2 genomes are allowed only marginal leeway to evolve. This should not come as a surprise, as every part of its genome is exploited on multiple levels: the stem–loop and adjacent motifs need intense conservation for (i) binding and cleavage by the replication complex (Steinfeldt et al., 2001); (ii) the Cap mRNA splicing acceptor site (Mankertz et al., 1998b); and (iii) the larger part of the Rep promoter sequence (Mankertz and Hillenbrand, 2002). The region clockwise of the stem–loop cannot be tampered with as its DNA encodes the replication protein Rep and its frame-shifted splice variant Rep' (Mankertz and Hillenbrand, 2001) as well as ORF3 (Liu et al., 2005) and ORF4 (He et al., 2013) on the amino acid level. On a nucleotide level, this stretch also contains sequences for the Rep' mRNA splicing donor and acceptor sites (Mankertz and Hillenbrand, 2001), and the Cap mRNA splicing donor site and the Cap promoter (Mankertz and Hillenbrand, 2002; Mankertz et al., 1998b). The genomic part counterclockwise to the stem–loop is suggested to have more freedom for variation because it only encodes Cap at the amino acid level (Nawagitgul et al., 2000) and the smaller part of the Rep promoter (Mankertz and Hillenbrand, 2002) on the nucleotide level. This understanding makes any conserved sequence of ORF2 an interesting target for investigation.
part in a larger constellation of positively charged amino acids comprising the basic sequence \textit{RIRKVK}^{102}, our understanding was that these basic amino acids stabilize the negatively charged viral ssDNA strand upon encapsidation. That this stretch of basic residues is also implicated in Cap mRNA translation (Figs. 2 and 5) came as a complete surprise and the reason for this still mystifies us. It is hard to craft an explanation without falling into a chicken or egg causality dilemma. One might think that Cap mRNA needs the Cap protein to be exported efficiently from the

Fig. 5. (a) Cap mRNA copy number kinetics. The figure represents three independent single experiments. (b) Immunostaining of Cap-positive parental clone-transfected and M4-transfected PK15-cells. Magnification, \( \times 20 \). (c) Virus titres of the synonymous M4 mutant and parental virus in supernatants collected at different time points after transfection. (d) Virus titres of the synonymous M4 mutant and parental virus at different passages. Results in (a), (c) and (d) are shown as means \( \pm SD \).
nucleus, but for Cap to come into existence an efficient viral mRNA export would be required first. Likewise, the Cap protein might act as a molecular switch turning on the Cap translation machinery, but this would require prior translation of Cap. We tried to solve this ‘catch 22’ situation by co-transfecting with a WT PCV2 strain, but even then we saw a preferential translation of WT PCV2 (Fig. 6). This argues in favour of a vision where the basic amino acid sequence is not so much a biological switch enhancing translation but rather takes part in a molecular interface interacting with another factor. One can imagine that the basic amino acids of the capsid protein have to be shielded from non-specific interactions with other proteins and nucleic acids, before being imported to the nucleus where they are deposited on viral DNA with high precision. In many ways, they resemble histones that are safeguarded and escorted by histone chaperones. Interestingly, an interaction between Cap and the histone chaperones NAP1 and NPM has been described (Finsterbusch et al., 2009). It is tempting to think that the removal of the positively charged amino acid pattern in the M1, M2 and M3 Cap proteins abolishes their interaction with co-translational chaperones, causing any further Cap mRNA translation to grind to a halt and leaving nascent Cap polypeptides stuck on the negatively charged ribosome. In this respect, the partial rescue of mutant Cap translation that we saw by co-transfecting with a WT PCV2a virus (Fig. 6) may be caused by a mutant Cap–WT Cap interaction that releases the mutant Cap from the ribosome and allows translation to continue, albeit still inefficiently. PCV1 Cap proteins display a virtually identical 98RIRKVK102 sequence, and it would be interesting to see whether M3 could still be rescued after co-transfection with a PCV1 Cap expression construct. PCV1 Cap proteins are expected to have inferior binding capabilities to PCV2 Cap proteins because of their antigenic differences. PCV1 should therefore not be able to restore M3 expression if rescue is indeed dependent on an interaction with WT Cap. In addition, an unbiased small-interfering RNA screen targeting histone chaperones could identify which chaperones, if any, are involved in PCV2 Cap translation.

Fig. 6. Left column: single transfection with PCV2b-based mutants showing the typical mutant Cap expression pattern. Middle column: co-transfection of the PCV2b-based mutants with a PCV2a virus strain rescues mutant Cap expression. Right column: co-transfection does not affect PCV2a Cap expression. Magnification, × 20.
As a final remark, it is conceivable that the replication protein itself exerts some additional chaperone activity. Rep may just guide Cap subunits to the right subnuclear compartment for further assembly or, even more speculatively, Rep might simply drive genome encapsidation. As a matter of fact, all circovirus replication proteins share a common superfamily 3 helicase domain (Roux et al., 2013), which assembles into ring-shaped multimers responsible for unwinding dsDNA by means of an ATPase-powered molecular motor (Hickman and Dyda, 2005). Should this activity be performed in close proximity to capsid proteins, it is not improbable that Rep subunits form replicative complexes displacing the viral strand towards adjacent capsid subunits, thus facilitating the process of genome packaging in complete harmony with replication. Hypothetical as this may be, an elegant relationship also exists in the closely related beak and feather disease virus between the two viral proteins, where Rep nuclear localization depends entirely on a cytoplasmic interaction with Cap, after which both shuttle to the nucleus (Heath et al., 2006). These findings all support a view for circoviruses in which an intimate relationship exists between Cap and Rep that has been underestimated until now, and that may serve as a working model for future experimental studies.

In conclusion, this study showed that the conserved stretch of basic residues comprising 97RIRVK102 of PCV2 Cap is crucial for its expression. Mutations in this pattern resulted in: (i) reduced translation of Cap; (ii) hindered Cap/Rep co-localization; and finally (iii) unfruitful PCV2 virions. Additionally, co-transfection with a WT PCV2 virus partially rescued mutant Cap expression, highlighting the importance of these amino acids for efficient translation of Cap mRNA into protein. We speculate that 97RIRVK102 is part of a molecular interface interacting with co-translational chaperones; however, future experiments need to be carried out to confirm this hypothesis.

**METHODS**

**Virus, cells and virus titration.** PCV2 strain 48285 (GenBank accession no. AF053394) clone 48285-24, PK-15 cells and culture conditions were described previously (Saha et al., 2012b). PCV2 strain LG (GenBank accession no. HM038034) clone pMD18/PCV2a-LG has been described elsewhere (Huang et al., 2011b). Virus titration was performed as described previously (Meerts et al., 2005).

**PCV2 mAbs.** Cap-binding mouse mAbs 38C1, 12E12 and 6E9 were generated in our laboratory and have been described elsewhere (Huang et al., 2011b). All antibodies were incubated for 1 h at 37°C. FITC-labelled goat anti-mouse IgG (Molecular Probes) was used as the primary and secondary antibodies, respectively. Images were obtained with a Leica TCS SP2 confocal microscope (Leica Microsystems). For double immunofluorescence staining for Cap and Rep, transfected PK-15 cells were first incubated with anti-Cap mAb 12E12 (diluted 1:2) and then with Alexa Fluor 594-conjugated goat anti-mouse IgG2b (1:200; Molecular Probes). Afterwards, the cells were stained for PCV2 Rep by incubation with anti-Rep mAb F210 D6 (1:500). Subsequently, a 1:200 dilution of FITC-labelled goat anti-mouse IgG1 (Molecular Probes) was added. All antibodies were incubated for 1 h at 37°C. The cells were washed three times with PBS after incubation with the primary and secondary antibodies. The specificity of the staining for different proteins was demonstrated using an irrelevant, isotype-matched mAb, IIIH4-5G (Costers et al., 2010), and III-D2 (Nauwynck and Pensaert, 1995), and by the complete absence of Cap- and Rep-specific fluorescence in mock-transfected PK-15 cells. The co-localization of Cap and Rep was analysed using the software CoLocalizer Pro 2.7.1 as described previously (Zinchuk and Zinchuk, 2008). Background correction of the images was performed prior to coefficient calculation. The co-localization rates were measured based on Manders’ coefficient, which varies from 0 to 1. A coefficient value of 0–0.6 indicates absence of co-localization, while a value of 0.6–1.0 indicates co-localization.

**Western blot analysis.** Western blot analysis was used to quantify the amount of Cap produced by the different mutants and the parental clone. Briefly, M1-, M2-, M3-, parental clone- and mock-transfected PK-15 cells were harvested at 36 h.p.t. SDS-PAGE and Western blotting were performed as described previously (Lefebvre et al., 2008). Anti-PCV2-Cap mAb 2E8 (Guo et al., 2011; Huang et al., 2011a), a PCV2-irrelevant mAb, 1C11 (Nauwynck and Pensaert, 1995), HRP-conjugated α-tubulin (Cell Signalling Technology) and HRP-conjugated sheep anti-mouse IgG(H+L) (Invitrogen) were used as the primary antibody, negative control, loading control and secondary antibody, respectively. Antigen–antibody complexes were visualized using an enhanced chemiluminescence assay (Amersham Biosciences).
Capture-ELISA. To quantify PCV2 Cap in the supernatants collected at different time points after transfection, a capture-ELISA was performed as described previously (Huang et al., 2011a) with minor modifications. Purified mAb 38Cl (5 μg ml⁻¹), biotin-labelled 38Cl (1:1000) and streptavidin-biotinylated HRP complex (1:1000; GE Healthcare) were used as coating, detecting and secondary antibody, respectively. The colour was developed and the reaction was stopped as described elsewhere (Saha et al., 2012a). The A500 was measured using a microplate reader (Bio-Rad). The capture-ELISA was performed in triplicate. The mean A500 value of the mock plus 3 SD was calculated to use as the cut-off value of the capture-ELISA.

RT-qPCR. Total RNA was extracted from transfected cells using an RNasy Mini kit (Qiagen). For each sample, 500 ng RNA was reverse transcribed into cDNA using a SuperScript First-Strand Synthesis System (Invitrogen).

The forward primer (5′-AGATGCGATTGCTCTTCAC-3′) and reverse primer (5′-GGGCTGACACATCGTTGCCGTA-3′) were designed for quantifying Cap mRNA transcripts were adapted from Yu et al. (2009). The reverse primer was designed to span the splice junction to ensure that PCV2 DNA was not amplified. qPCR was performed with an Applied Biosystems StepOnePlus Real-Time PCR System (Genetic Systems Co.) using SensiMix SYBR mastermix (Bioline).

ACKNOWLEDGEMENTS

We would like to thank Carine Boone and Lieve Sys for their excellent technical assistance. This study was supported by the National Natural Science Foundation of China (grant no. 31302110).

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