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Expression and processing of the canine calicivirus capsid precursor

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The ORF2 product of canine calicivirus (CaCV) was identified and its processing in mammalian cells was analysed. Immunoblot analysis revealed the presence of the 75 kDa capsid precursor in addition to a 57 kDa capsid protein and a 22 kDa N-terminal polypeptide in CaCV-infected cells treated at an elevated temperature. When the CaCV ORF2 was expressed in a transient mammalian expression system, only the 75 kDa precursor was detected in immunoblot analysis, suggesting that no post-translational processing occurred in this system. However, the precursor was processed to a 57 kDa protein and a 22 kDa polypeptide by the proteinase of feline calicivirus (FCV) when this was co-expressed with ORF2. Processing was blocked by site-directed mutagenesis of the putative cleavage site in the capsid precursor. The results indicate that the proteinase of FCV can cleave the capsid precursor of CaCV to produce the mature capsid protein and that CaCV may have a similar proteinase.

Canine calicivirus (CaCV) No. 48 strain was isolated from a domestic dog with fatal diarrhoea (Mochizuki *et al.*, 1993). Although the virion, antigenic and biological properties of CaCV No. 48 strain are similar to those of the first CaCV strain described by Schaffer *et al.* (1985), very little is known about genome organization and replication. Recently, the presence of three open reading frames (ORFs) in the genome of CaCV No. 48 strain was demonstrated by sequence analysis of a region from the RNA polymerase to the 3′ poly(A) tail of its genome (Roerink *et al.*, 1999). It was suggested that ORF2 encoded the capsid precursor as its deduced amino acid sequence had relatively high similarity to that of feline calicivirus (FCV) and San Miguel sealion virus (SMSV). In this study, the product of ORF2 was identified and its processing was analysed by co-expression of CaCV ORF2 and the putative proteinase region in FCV ORF1 in COS-7 cells.

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CaCV No. 48 strain was propagated in Miyazaki University canine mammary gland mixed tumour (MCM-B2) cells (Prio soeryanto *et al.*, 1995). In order to inhibit proteolytic processing, CaCV-infected cells were treated at an elevated temperature of 45 °C as previously described (Carter, 1989; Shin *et al.*, 1993).

A serum monospecific to the capsid protein was obtained from BALB/c mice that had been immunized with approximately 10 μg/mouse CaCV capsid protein. The capsid protein used for the immunization was prepared as follows. CsCl-purified virus was separated by 10% SDS–PAGE and stained with Coomassie brilliant blue R250 as described previously (San Gabriel *et al.*, 1997). The band of the capsid protein was sliced and eluted in an electro-eluter (Model 422; Bio-Rad). The eluted protein was precipitated with acetone and resuspended in PBS for future use.

The CaCV ORF2 expression plasmid was constructed as follows. CaCV RNA was extracted from the purified CaCV suspension as previously described (Roerink *et al.*, 1999). Reverse transcription was carried out using oligo(dT) primer. A cDNA fragment was produced by PCR using synthetic oligonucleotides CaCV1 (5′ GAACTCGAGATGGCTCGC TATCTTGAACT 3′) and CaCV2 (5′ GCTCTAGATCAT AGTGTTGTAGCGCTAC 3′) as primers. Primer CaCV1 corresponded to nt 1–20 of the CaCV ORF2 with a *Xho*I restriction enzyme site upstream from the first ATG initiation codon (underlined), and primer CaCV2 was complementary to nt 2057–2076 of the CaCV ORF2 and contained an *Xba*I restriction enzyme site. The PCR-amplified fragment was digested by *Xho*I and *Xba*I, and cloned between the *Xho*I and *Xba*I sites of the pME18S expression vector (Shin *et al.*, 1993). The constructed plasmid was designated pDCV-II, and contained the first ATG of ORF2 directly under the control of

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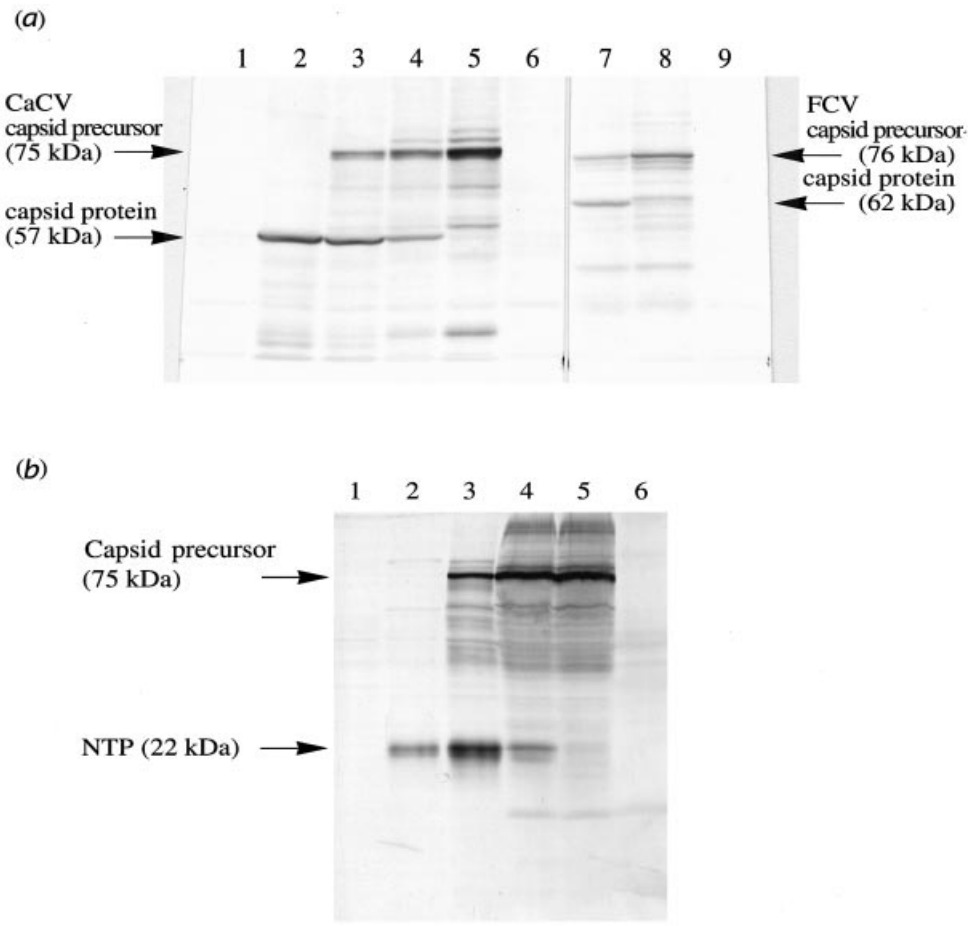

Fig. 1. Immunoblot analysis of the capsid precursor and proteolytic processing of the capsid protein of CaCV. Infected cells and transfected cells were solubilized and electrophoresed in 7·5 % (*a*) and 15% (*b*) polyacrylamide gels. The resolved proteins were electro-transferred onto membranes. Blots were detected with serum monospecific to the CaCV capsid protein (*a*, lanes 1–6), a MAb to the FCV capsid protein (*a*, lanes 7–9) and anti-GST/NTP serum (*b*). Lanes: 1, mock-infected MCM-B2 cells; 2, CaCV No. 48-infected MCM-B2 cells; 3, MCM-B2 cells infected with CaCV No. 48 and treated at 45 °C; 4–9, COS-7 cells transfected with plasmids, pDCV-II and pMCV-3C (lane 4), pDCV-II and pME18S (lane 5), pMCV-3C (lanes 6 and 9), pMCV-II and pMCV-3C (lane 7), pMCV-II and pME18S (lane 8).

the SRα promoter (Takebe *et al.*, 1988). pMCV-3C, which expresses the putative 3C region of FCV strain F4 (Oshikamo *et al.*, 1994), was constructed as follows. A cDNA fragment including the putative 3C region was generated by PCR with synthetic oligonucleotides XHO-ATG5′3C (5′ AATCTCG-AGATGT CTGGGCCTGGCACTAA 3′) and PST-TCA-3′3C (5′ GGCTGCAGTCATTCCAAGAAGATGTTCAT 3′) as primers and a plasmid containing the ORF1 of FCV F4 as template. Primer XHO-ATG5′3C corresponded to nt 3214–3230 of the FCV ORF1 with a *Xho*I restriction enzyme site and an initiation codon, and primer PST-TCA-3′3C was complementary to nt 4018–4035 of the FCV ORF1 and contained a *Pst*I restriction enzyme site and a termination codon. The PCR-amplified fragment was digested by *Xho*I and *Pst*I, and cloned between the *Xho*I and *Pst*I sites of pME18S. The FCV ORF2 expression vector pMCV-II was constructed as described previously (Shin *et al.*, 1993). Three micrograms of pDCV-II or pMCV-II and 1 μg pMCV-3C or pME18S were co-transfected into COS-7 cells according to previously described methods with minor modifications (Seed & Aruffo, 1987; Shin *et al.*, 1993).

Detection and identification of the CaCV capsid precursor were performed by immunoblot analysis using the serum monospecific to the capsid protein (Fig. 1*a*, lanes 1–3, and 5). The serum showed specific reactivity with 57 kDa capsid protein in CaCV-infected cells (Fig. 1*a*, lane 2). In CaCV-infected cells, which were treated at an elevated temperature of 45 °C, 75 kDa and 57 kDa proteins were specifically detected (Fig. 1*a*, lane 3). This phenomenon was also seen in similar experiments using SMSV- and FCV-infected cells (Fretz & Schaffer, 1978; Carter, 1989; Shin *et al.*, 1993). The 75 kDa protein was identified as the precursor of the 57 kDa CaCV capsid protein in this study. When the ORF2 of CaCV was expressed transiently in COS-7 cells by transfection with

pDCV-II, the 75 kDa protein was detected in the immunoblot analysis (Fig. 1*a*, lane 5). The additional bands above the 75 kDa protein may be polypeptides translated by incorrect initiation from an ATG codon in-frame and upstream from the first ATG of CaCV ORF2 or by read-through of the termination. In addition, low density minor bands were considered to be degraded products of the capsid precursor as described previously (Shin *et al*., 1993). The molecular mass of the 75 kDa protein detected in the pDCV-II-transfected cells was similar to that expected if the CaCV ORF2 was translated from the first ATG in ORF2 to its termination codon (i.e. 76 180 Da). The mobility of the 75 kDa protein in the transfected cells was identical to that of the capsid precursor (Fig. 1*a*, lanes 3 and 5). The expressed protein therefore seems to have the same basic characteristics as the CaCV capsid precursor both in antigenicity and in electromobility. This result also suggests that no post-translational processing by autocatalytic or host cell-mediated cleavage has occurred in this system.

It has been reported that FCV-encoded proteinase mediates the cleavage of the capsid precursor (Sosnovtsev *et al*., 1998). We examined whether a polypeptide of the putative 3C proteinase region is active in our expression system using pMCV-3C (Fig. 1*a*, lanes 7–9). In COS-7 cells which were co-transfected with pMCV-3C and pMCV-II, the 62 kDa capsid protein of FCV, in addition to the 76 kDa capsid precursor, was detected by immunoblot analysis using a MAb against the capsid protein (Fig. 1*a*, lane 7). As the transiently expressed proteinase in this study was confirmed to be active, proteolytic processing of the CaCV capsid protein was analysed by immunoblotting with COS-7 cells co-transfected with pDCV- II and pMCV-3C (Fig. 1*a*, lane 4). The 75 kDa and 57 kDa proteins were detected in the co-transfected COS-7 cells. The molecular mass of the detected proteins in the co-transfected COS-7 cells was equivalent to that of the corresponding capsid protein and precursor which were detected in the CaCV- infected cells (Fig. 1*a*, lanes 3 and 4). This showed that the 75 kDa CaCV precursor was processed to a 57 kDa protein by the co-expressed proteinase of FCV in the COS-7 cells.

In FCV, the capsid precursor is cleaved by a virus-encoded proteinase between amino acid positions 124 and 125 (Glu_{124} and Ala_{125}) (Sosnovtsev *et al*., 1998), and the 14 kDa N-terminal polypeptide was identified in infected cells (Tohya *et al*., 1999). In CaCV, a corresponding cleavage site (Glu_{157}/Ser_{158}) was suggested by homology analysis with FCV and SMSV (Roerink *et al*., 1999). In order to determine whether the FCV proteinase cleaved at the corresponding site in the CaCV capsid precursor, we prepared a serum against an N-terminal polypeptide (NTP) corresponding to amino acids Met_1 to Glu_{157} of the capsid precursor using a glutathione *S*-transferase (GST)/NTP fusion protein. The GST/NTP fusion protein expression plasmid was constructed as follows. The 5′-end 471 nt region of ORF2 encoding the NTP was PCR-amplified using plasmid pDCV-II as the template and synthetic oligonucleotides CaCV ORF2-A (CTCAGGATCCAGATG GCTCGCTATCTTGAA) and CaCV ORF2-AR (ATACT CGAGTTCCGCGCGGAATTGGAATTC) as primers. Primer CaCV ORF2-A corresponded to nt 1–18 of the CaCV ORF2 with a *Bam*HI restriction enzyme site and additional nucleotides (AG) upstream of the ATG initiation codon to adjust the frame, and CaCV ORF2-AR was complementary to nt 451–471 of the ORF2 and contained a *Xho*I restriction enzyme site. The PCR-amplified fragment was cloned between the *Bam*HI and *Xho*I sites of the vector pGEX-5X-1 (Pharmacia Biotech). Expression and purification of the fusion protein and immunization of mice were performed as described previously (Tohya *et al*., 1999).

The results of immunoblot analysis using the anti-GST/NTP serum are shown in Fig. 1(*b*). The serum showed specific reactivity with a 22 kDa polypeptide in the CaCV-infected cells (Fig. 1*b*, lane 2). The anti-GST serum prepared as a control showed no specific reactivity with the proteins in the cells (data not shown). In addition to the 22 kDa polypeptide, the 75 kDa capsid precursor was detected in CaCV-infected cells which were treated at the elevated temperature (Fig. 1*b*, lane 3), indicating that the cleaved N-terminal part of the capsid precursor has a molecular mass of 22 kDa, although the molecular mass of the 22 kDa polypeptide was a little larger than the mass of 18 256 Da deduced from sequence analysis. The 22 kDa polypeptide, in addition to the 75 kDa capsid precursor, was also detected only in the pDCV-II and pMCV-3C co-transfected COS-7 cells, and not in the pDCV-II and pME18S co-transfected cells (Fig. 1*b*, lanes 4 and 5). The result suggested that the proteinase of FCV cleaves at the authentic site in the capsid precursor of CaCV and that CaCV may have a similar proteinase possibly encoded in its ORF1.

A feature of the FCV proteinase is its high substrate specificity that is determined by the structure of the cleavage site sequence (Sosnovtsev *et al*., 1998). In order to confirm the suggested cleavage site (Glu_{157}/Ser_{158}), we investigated whether processing of the CaCV capsid precursor could be blocked by the co-expression system. The *Xho*I/*Eco*RV fragment (924 bp) containing the 5′-end of ORF2 was excised from pDCV-II and ligated into pBluescript II KS(+) (Stratagene). The Glu_{157} mutation (GAA to AAA for Lys) and the Ser_{158} mutation (TCC to CCC for Pro) were introduced, respectively, into the fragment using mutagenic oligonucleotides designed to create substitutional mutations and the TaKaRa LA PCR *In Vitro* Mutagenesis kit (TAKARA) according to the manufacturer's instructions. After both mutations in the fragments were confirmed by sequence analysis, the mutated fragments were used to replace the parent fragment in pDCV-II. The mutated expression plasmids were designated pDCV-II mut EK and pDCV-II mut SP for the Glu_{157} and Ser_{158} mutations, respectively. In the COS-7 cells co-transfected with the mutated plasmids and the pMCV-3C, the mature 57 kDa capsid protein was not detected by

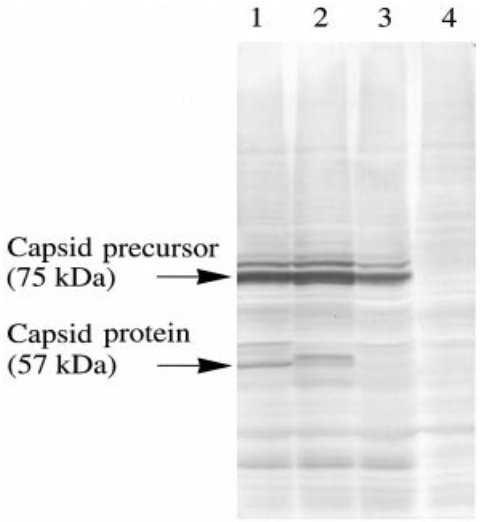

Fig. 2. Analysis of the cleavage site in the CaCV capsid precursor by site-directed mutagenesis. The mutated capsid precursor was co-expressed with the proteinase of FCV in COS-7 cells. The expressed polypeptides were detected by immunoblot analysis using a serum monospecific to the CaCV capsid protein. COS-7 cells were transfected with the following plasmids: pDCV-II and pMCV-3C (lane 1); pDCV-II mut EK and pMCV-3C (lane 2); pDCV-II mut SP and pMCV-3C (lane 3); and pMCV-3C (lane 4).

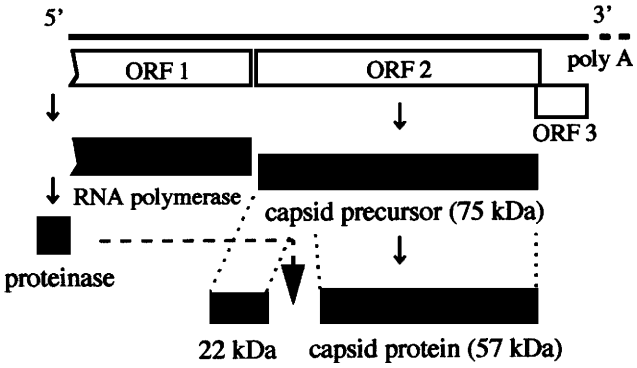

Fig. 3. Expression and processing of the capsid precursor of CaCV. The top line indicates the region from the RNA polymerase to the 3′ poly(A) tail of CaCV RNA genome, the sequence of which has been reported (Roerink *et al.*, 1999). The ORFs are shown as open boxes and polypeptides translated from each ORF are shown as filled-in boxes. In ORF1, only the RNA polymerase region is shown, because the upstream part of the ORF1 sequence is unknown. The capsid precursor translated from ORF2 would be cleaved by proteinase that might be translated from a region upstream of the RNA polymerase in ORF1 and processed into the mature capsid protein and a 22 kDa N-terminal polypeptide.

immunoblot analysis using the serum monospecific to the capsid protein (Fig. 2, lanes 2 and 3). The result shows that processing by the FCV proteinase was blocked by changing the amino acids at the site and strongly suggests that the Glu_{157}/Ser_{158} site is the cleavage site in the CaCV capsid precursor by the virus-encoding proteinase.

In the members of *Caliciviridae*, only FCV and SMSV have been known to produce capsid precursor proteins (Carter, 1989; Fretz & Schaffer, 1978). Their ORF2s are larger than the corresponding regions for the capsid genes of other caliciviruses (Neill, 1992). The *Caliciviridae* study group of the International Committee on Taxonomy of Viruses (Pringle, 1998) has proposed a new genus, *Vesivirus*, with type species swine vesicular exanthema virus. Sequence analysis suggests that FCV and SMSV would be classified in this genus with newly analysed CaCV, which also has a larger ORF2 (Roerink *et al.*, 1999). In this study, the 75 kDa capsid precursor was identified as the translational product from ORF2 of CaCV and its cleavage by a viral proteinase was shown. Following the cleavage of the capsid precursor, the C-terminal part forms the mature 57 kDa capsid protein and the N-terminal part forms the 22 kDa polypeptide (Fig. 3). As the system for expression and processing of the capsid precursor of CaCV revealed in this study resembled that of the FCV system (Sosnovtsev *et al.*, 1998; Tohya *et al.*, 1999) and the FCV proteinase was compatible in the CaCV system, similar systems for ORF2 expression may be adopted by caliciviruses in the genus *Vesivirus*.

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