Short Communication

Analysis of protein–protein interactions in the feline calicivirus replication complex

William J. Kaiser,[1] Yasmin Chaudhry,[1] Stanislav V. Sosnovtsev[2] and Ian G. Goodfellow[1]†

[1]School of Biological Sciences, University of Reading, Whiteknights, Reading RG6 6AJ, UK

[2]Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

Correspondence
Ian G. Goodfellow
I.Goodfellow@ic.ac.uk

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Caliciviruses are a major cause of gastroenteritis in humans and cause a wide variety of other diseases in animals. Here, the characterization of protein–protein interactions between the individual proteins of *Feline calicivirus* (FCV), a model system for other members of the family *Caliciviridae*, is reported. Using the yeast two-hybrid system combined with a number of other approaches, it is demonstrated that the p32 protein (the picornavirus 2B analogue) of FCV interacts with p39 (2C), p30 (3A) and p76 (3CD). The FCV protease/RNA polymerase (ProPol) p76 was found to form homo-oligomers, as well as to interact with VPg and ORF2, the region encoding the major capsid protein VP1. A weak interaction was also observed between p76 and the minor capsid protein encoded by ORF3 (VP2). ORF2 protein was found to interact with VPg, p76 and VP2. The potential roles of the interactions in calicivirus replication are discussed.

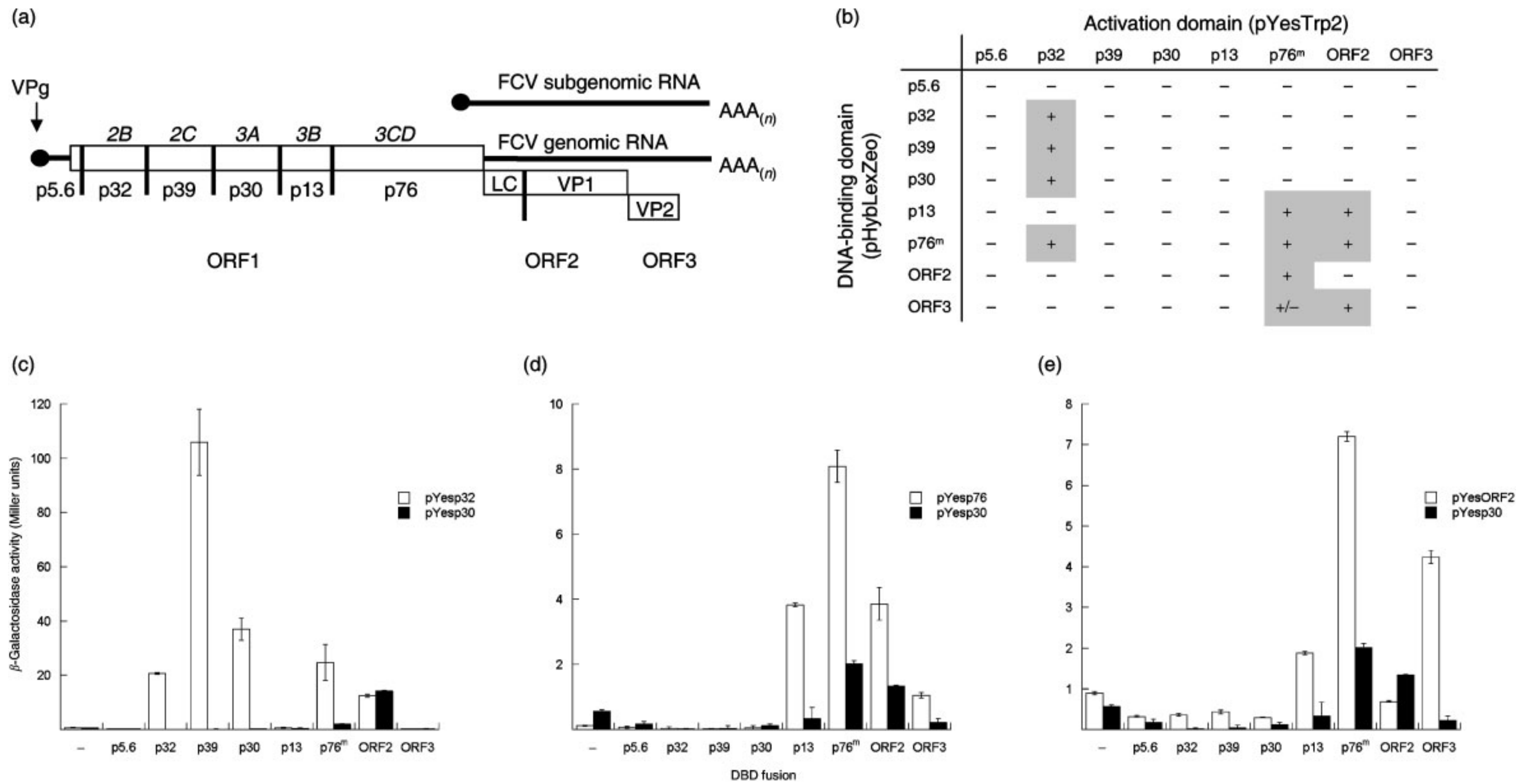
Caliciviruses are a family of positive-stranded RNA viruses that cause gastroenteritis in humans and a number of debilitating diseases in animals. The human enteric caliciviruses are considered as the major cause of viral gastroenteritis in the developed world and have been associated with over 85 % of non-bacterial gastroenteritis outbreaks in Europe during 1995–2000 (Lopman *et al.*, 2003). Despite valiant efforts to culture these viruses (Duizer *et al.*, 2004), there has been no successful cultivation of a human calicivirus to date, although recent reports have demonstrated replication and packaging of norovirus RNA in cell culture (Asanaka *et al.*, 2005). As a result, the study of molecular mechanisms of calicivirus replication has been hampered.

Replication of positive-stranded RNA viruses occurs on the surface of membranous vesicles in the cytoplasm of the infected cell (Wimmer *et al.*, 1993; Schaad *et al.*, 1997; Chen & Ahlquist, 2000; El-Hage & Luo, 2003). The formation of these replication complexes occurs via a complex network of protein–protein interactions between individual viral proteins, and also between viral and host-cell proteins. Characterization of interactions between viral proteins using the yeast two-hybrid system (Fields & Song, 1989) has allowed the formation of protein linkage maps for a number of viruses, including *Poliovirus* (Cuconati *et al.*, 1998; Xiang *et al.*, 1998), *Potato virus A*, *Pea seed-borne mosaic virus* (Guo *et al.*, 2001), *Infectious bursal disease virus* (Tacken *et al.*, 2000), *Vaccinia virus* and a number of others (reviewed by Uetz *et al.*, 2004). Although not exhaustive, such linkage maps are informative, as they identify not only new interactions, but also those that are amenable to fine-detail mapping using yeast two-hybrid analysis. This can often allow the identification of the specific amino acids or exposed surfaces of proteins that are required for the interactions to occur, information that is useful for the design of small-molecule inhibitors of such interactions.

In contrast to the human caliciviruses, *Feline calicivirus* (FCV), porcine enteric calicivirus (Chang *et al.*, 2002) and the recently identified murine norovirus 1 (Wobus *et al.*, 2004) can be propagated in culture and, as such, provide good model systems with which to study calicivirus biology. FCV is a particularly good model, given the speed with which the virus replicates and the availability of a reverse-genetics system (Sosnovtsev & Green, 1995). Studies have shown that the proteins encoded by FCV are associated with membrane-bound replication complexes (Green *et al.*, 2002). However, studies characterizing the network of protein–protein interactions that occur in these complexes are lacking. To begin to identify a number of these interactions, we have used the yeast two-hybrid system to detect interactions between the various proteins of FCV, as a model system for the other members of the family *Caliciviridae*.

Coding regions from the experimentally derived FCV cleavage map (Fig. 1a) (Sosnovtsev *et al.*, 2002) were amplified by PCR and cloned into both the LexA DNA-binding domain (DBD) fusion plasmid pHybLexZeo and the pYesTrp2 Gal4 transcriptional-activation domain (AD)

†Present address: Department of Virology, Faculty of Medicine, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, UK.



DBD \ AD	p5.6	p32	p39	p30	p13	p76m	ORF2	ORF3
p5.6	–	–	–	–	–	–	–	–
p32	–	+	–	–	–	–	–	–
p39	–	+	–	–	–	–	–	–
p30	–	+	–	–	–	–	–	–
p13	–	–	–	–	–	+	+	–
p76m	–	+	–	–	–	+	+	–
ORF2	–	–	–	–	–	+	–	–
ORF3	–	–	–	–	–	+/–	+	–

Fig. 1. Identification of calicivirus protein–protein interactions. (a) Graphic representation of the experimentally derived FCV cleavage map. Text in italics represents the name of picornavirus analogues. LC represents the leader capsid sequence. (b) Summary of the interactions characterized. Positive interactions are shaded. (c–e) Quantification of positive interactions observed between individual FCV proteins. Yeast extracts from cells containing both DBD- and AD-fusion partners were prepared and the levels of β-galactosidase are expressed in Miller units.

fusion plasmid (Invitrogen). This system was chosen due to the ability to regulate the expression of the AD-fusion protein by using a galactose-inducible promoter, a feature that has previously been beneficial in similar studies on poliovirus protein–protein interactions (Xiang *et al.*, 1998).

Preliminary results indicated that expression of wild-type FCV p76 protease/RNA polymerase (ProPol) in yeast was toxic after induction (data not shown). Toxicity was probably due to unregulated proteolytic activity degrading essential yeast proteins, possibly eIF4G and PABP, as observed previously during FCV infection of feline kidney cells (Kuyumcu-Martinez *et al.*, 2004; Willcocks *et al.*, 2004). A proteolytically inactive form of p76 ($p76^m$) (Wei *et al.*, 2001), in which the codon encoding catalytic Cys-1193 was changed to one encoding a glycine, had a less-significant effect on the growth of transformed yeast strains (data not shown) and was therefore used for subsequent analysis.

All media, buffers and methods for the yeast two-hybrid system were as described in the Dual Bait Hybrid Hunter manual (Invitrogen) and the yeast protocols manual (Clontech). DBD- and AD-fusion constructs were transformed into the yeast reporter stain EGY48/pSH18-34 (Invitrogen) by using lithium acetate. Expression of each fusion was confirmed by using Western blot analysis with antibodies directed towards either the DBD present in pHybLexZeo or the SV5 epitope present in pYesTrp2 (data not shown). To detect interactions between individual viral proteins, β-galactosidase assays were carried out on extracts from strains containing both DBD- and AD-fusion plasmids in all possible combinations. In addition, all fusion plasmids were tested in the presence of vectors encoding the DBD and AD alone to control for non-specific activation (data not shown). Positive interactions were identified as those that reproducibly gave β-galactosidase activity greater than that observed by using either DBD or AD alone as the interacting partner (Fig. 1b). $p76^m$– and ORF2–DBD fusions were found to activate transcription to detectable levels in the absence of an AD-fusion partner (data not shown). Positive interactions with $p76^m$ and ORF2 were therefore identified by their ability to reproducibly give higher levels of activation than AD alone (data not shown) or a p30–AD fusion, as highlighted in Fig. 1. The levels of activation observed with pYesp30, expressing a p30–AD fusion, are representative of the levels obtained with p5.6–AD, p13–AD, p39–AD, ORF3–AD and AD alone fusion partners (data not shown).

Many of the interactions observed appeared to be 'unidirectional', e.g. $p76^m$ interacted with p32 when present as the DBD partner, but not when present as the AD partner (Fig. 1b–d). This unidirectionality has previously been observed in similar studies (Cuconati *et al.*, 1998) and is likely to reflect conformational constraints of the interactions. Variation in the β-galactosidase levels were observed from day to day; however, when compared to internal controls, the relative levels of each interacting pair were consistent (data not shown).

Interactions of the FCV p32 protein, the putative picornavirus 2B analogue, with the viral helicase/NTPase p39, the picornavirus 3A analogue p30 and the viral ProPol p76 were identified (Fig. 1c). In addition, a p32–p32 interaction was observed (Fig. 1c), suggesting the possible existence of multimeric forms of p32. Using deletion analysis, the C terminus of p32 (aa 206–285) was identified as the domain required for the observed interactions with p32, p30, p39 and $p76^m$ (Fig. 2a). Similar deletion analysis of the helicase/NTPase p39, the picornavirus 2C analogue, indicated that the N-terminal 80 aa are required and sufficient for the observed p32–p39 interaction (Fig. 2b).

To confirm the ability of p32 to form multimeric complexes, human embryonic kidney (293T) cells were transfected with a plasmid expressing FCV p32. Lysates were prepared by resuspending cells in lysis buffer [10 mM Tris

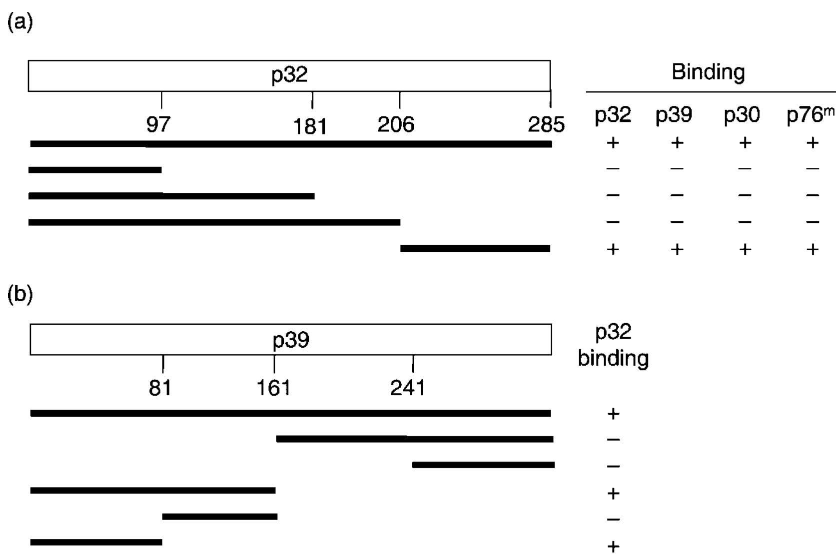


Fig. 2. Deletion analysis of p32 and p39. (a) N- and C-terminally truncated p32–AD fusions were tested for their ability to interact with p32–DBD, p39–DBD, p30–DBD and $p76^m$–DBD fusion partners. Interactions that repeatedly gave β-galactosidase levels greater than that of AD alone were scored as positive (+). (b) p39–DBD deletions were tested for their ability to interact with a p32–AD fusion partner as described above.

(pH 7·5), 10 mM NaCl, 2 mM EDTA, 0·5 % Triton X-100] 48 h post-transfection. Western blot analysis was carried out under both reducing and non-reducing conditions using anti-p32 antisera (Fig. 3a). FCV p32 was found to produce complexes of approximately twice the predicted molecular mass (64 kDa) under non-reducing conditions (Fig. 3a), confirming the existence of p32 dimers. The multiple reactive bands detected at the position equivalent to dimeric p32 are the likely results of heterogeneity in the intra- and inter-molecular disulphide bonds present in the dimeric complex.

The interaction of p32 with p30 was confirmed by co-transfecting 293T cells with a plasmid encoding p32 with either HA-tagged p30 or HA-tagged VP1. Sequences encoding the HA tags were added to the N terminus of each protein by PCR prior to cloning into pCI (Promega), containing a cytomegalovirus promoter. Forty-eight hours post-transfection, cells were lysed (see above) and immunoprecipitated with an anti-HA monoclonal antibody (mAb) (Sigma). Samples of the precipitated complex and cell lysate were analysed for the presence of HA-tagged proteins and p32 by Western blot (Fig. 3b). Whereas p32 was immunoprecipitated readily with anti-HA mAb when co-transfected with HA–p30, no p32 was present in the precipitated complex when co-transfected with HA–VP1. Similar levels of p32 were present in both extracts prior to co-immunoprecipitation (Fig. 3b), confirming a specific interaction between p32 and p30. Previous work has demonstrated that a 33 kDa protein can be immunoprecipitated from infected cells by using antisera to VPg (p13) (Fig. 5; Mitra *et al.*, 2004). It is likely this protein is in fact the FCV p32 protein that co-immunoprecipitated with the p30–VPg (p43) precursor found in high levels in infected cells. Work is currently under way to confirm that the p30-containing precursor p43 interacts directly with p32.

The function of p32 in FCV replication is yet to be determined; however, its position in the viral genome (Fig. 1a) indicates it to be a picornavirus 2B analogue. Previous work with the Norwalk virus p32 homologue (p48) has demonstrated a function in Golgi disassembly, which was not seen with FCV p32 (Fernandez-Vega *et al.*, 2004). A direct interaction of the Norwalk virus p48 with the SNARE regulator VAP-A has also been reported (Ettayebi & Hardy, 2003), but the role of this interaction in virus replication has yet to be determined. Preliminary work has indicated that p32 is an integral membrane protein (data not shown) and it is possible that p32 functions to form the 'core' of the replication complex, anchoring many of the other components in place. Work is under way to further characterize the role of p32 and the observed p32–viral-protein interactions in FCV replication.

The two-hybrid screen also revealed that the FCV ProPol $p76^m$ interacted with itself, VPg (p13) and ORF2, the region encoding the precursor of the capsid protein VP1 (Fig. 1b, d). A relatively poorly activating, but reproducible, interaction was observed with the minor capsid protein ORF3 (VP2), shown as +/− in Fig. 1(b). The existence of RNA polymerase oligomers in caliciviruses may highlight similarity to a number of other positive-stranded RNA viruses, including both *Poliovirus* (Pata *et al.*, 1995) and *Hepatitis C virus* (Wang *et al.*, 2002), where polymerase oligomerization has been shown to be biologically important. Indeed, the crystal structure of the RNA polymerase from *Rabbit hemorrhagic disease virus* (RHDV) identified contacts between two polymerase molecules present in the asymmetric unit (Ng *et al.*, 2002).

To confirm the interaction of p76 ProPol with VPg, purified recombinant proteins were used in an ELISA-based interaction assay as described previously (Goodfellow *et al.*, 2005).

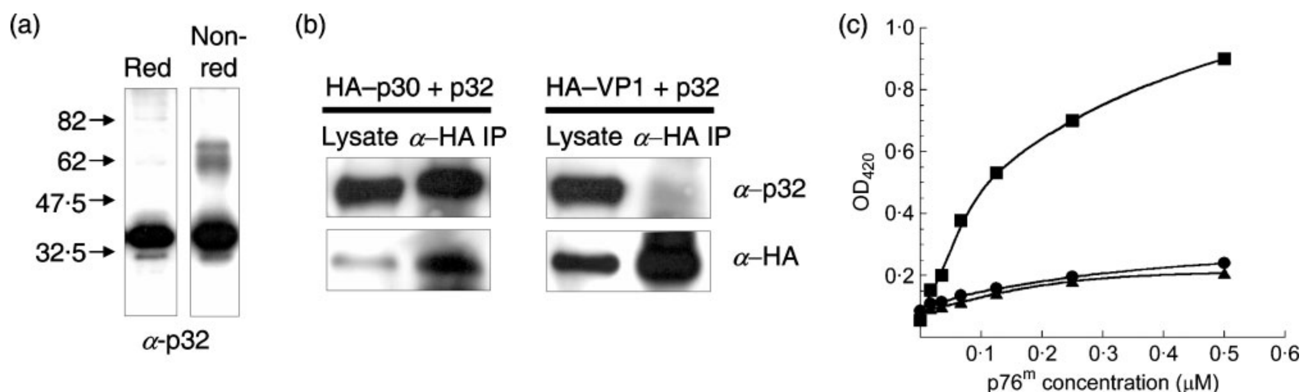


Fig. 3. Biochemical confirmation of protein–protein interactions. (a) Dimerization of the FCV p32 protein. Western blot analysis was carried out under both reducing and non-reducing conditions with anti-p32 antisera on lysates prepared from 293T cells expressing FCV p32. (b) Co-immunoprecipitation of p32 with HA-p30. Western blot analysis was performed on lysates and anti-HA mAb-immunoprecipitated complexes using either anti-HA mAb or anti-p32 antiserum. (c) ELISA capture of $p76^m$ on recombinant VPg. Maltose-binding protein (MBP; ▲), FCV VPg (■) or LDV VPg (●) was coupled to an ELISA plate and recombinant $p76^m$ was passed over. Capture of $p76^m$ was detected by using anti-p76 antiserum.

Untagged forms of FCV VPg, Lordsdale virus (a human calicivirus) VPg and FCV $p76^m$ were purified as described previously (Wei *et al.*, 2001; Goodfellow *et al.*, 2005). A 5 μg aliquot of purified FCV VPg, LDV VPg or maltose-binding protein (MBP) was coupled to an ELISA plate and serial dilutions of recombinant $p76^m$ were passed over. The interaction of $p76^m$ with immobilized VPg was detected by using antisera to $p76^m$ (Fig. 3c). Whereas both LDV VPg and MBP failed to retain detectable levels of FCV $p76^m$, wells coated with FCV VPg retained $p76^m$ in a dose-dependent manner (Fig. 3c), confirming a direct p76–VPg interaction.

The role of the identified p76–viral-protein interactions has yet to be determined; however, as p76 functions as both the viral protease and RNA polymerase (Wei *et al.*, 2001), it is likely to play key roles in virus replication and genome encapsidation. The interaction of VPg with p76 is likely to play a role in the initiation of calicivirus RNA synthesis, as VPg is linked covalently to the viral genomic and subgenomic RNA (Schaffer *et al.*, 1980; Herbert *et al.*, 1997). Studies with RHDV polymerase have demonstrated that the polymerase can transfer nucleotide to VPg (Machín *et al.*, 2001), suggesting a protein-primed mechanism of RNA synthesis, as demonstrated for *Poliovirus* (Paul *et al.*, 1998). Similarly, studies using reverse genetics suggest that Tyr-24 of FCV VPg is essential for viral infectivity (Mitra *et al.*, 2004).

The FCV ORF2 that encodes both the leader capsid (LC) and mature major capsid protein VP1 (Fig. 1a) was found to interact with VPg (p13), $p76^m$ and ORF3 (VP2) (Fig. 1b, e). Given that encapsidated viral RNA is linked covalently to VPg (Herbert *et al.*, 1997), it is possible that the observed ORF2–VPg and ORF2–$p76^m$ interactions play a role in the encapsidation of the viral RNA. The calicivirus minor capsid protein VP2 encoded by ORF3 is thought to be present in mature virions at approximately 1–2 copies (Sosnovtsev & Green, 2000). The observed ORF2–VP2 interaction is likely to play a role in the packaging of VP2; however, whether VP2 or VPg interacts with LC or VP1 remains to be determined. Although ORF3 is not required for the formation of virus-like particles (Jiang *et al.*, 1992), it is essential for virus viability (Sosnovtsev *et al.*, 2005), suggesting an additional role in virus replication.

Molecular characterization of calicivirus replication is very much in its infancy. The identification of viral protein–protein interactions in the calicivirus replication complex is the first step in understanding the complex network of interactions that lead to viral genome replication. We would predict that, in addition to the interactions identified above, other interactions between viral proteins exist that were not detected in our screen. Our inability to detect such interactions may be primarily due to conformational constraints of the interactions and has been observed in many other such studies (Uetz *et al.*, 2004). Indeed, preliminary analysis suggests that the FCV p39 protein can form oligomers, which do not form when p39 is fused to additional non-viral sequences (data not shown). The interactions identified above provide good targets for small-molecule inhibitors that may prove beneficial as antiviral agents for the control of diseases caused by this family of viruses.

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