Feline calicivirus replication: requirement for polypyrimidine tract-binding protein is temperature-dependent

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The interaction of host-cell nucleic acid-binding proteins with the genomes of positive-stranded RNA viruses is known to play a role in the translation and replication of many viruses. To date, however, the characterization of similar interactions with the genomes of members of the family Caliciviridae has been limited to in vitro binding analysis. In this study, Feline calicivirus (FCV) has been used as a model system to identify and characterize the role of host-cell factors that interact with the viral RNA. It was demonstrated that polypyrimidine tract-binding protein (PTB) interacts specifically with the 5′ sequences of the FCV genomic and subgenomic RNAs. Using RNA interference it was shown that PTB is required for efficient FCV replication in a temperature-dependent manner. siRNA-mediated knockdown of PTB resulted in a 15- to 100-fold reduction in virus titre, as well as a concomitant reduction in viral RNA and protein synthesis at 32°C. In addition, virus-induced cytopathic effect was significantly delayed as a result of an siRNA-mediated reduction in PTB levels. A role for PTB in the calicivirus life cycle was more apparent at temperatures above and below 37°C, fitting with the hypothesis that PTB functions as an RNA chaperone, potentially aiding the folding of RNA into functional structures. This is the first functional demonstration of a host-cell protein interacting with a calicivirus RNA.

INTRODUCTION

The interaction of cellular proteins with RNA sequences and structures plays a critical role in many aspects of the processing, localization and translation of host-cell mRNAs. Often, the RNA-binding proteins participating in these interactions are also used by viruses in their own translation and replication mechanisms (Bushell & Sarnow, 2002; Shi & Lai, 2005). Such RNA–protein interactions are often a major determinant of viral tropism, due to cell-specific expression of such factors (Gutierrez et al., 1997; Pilipenko et al., 2000). A large number of interactions between host-cell proteins and viral RNA structures have now been identified, with some of the best characterized interactions being those that regulate translation from viral internal ribosome entry site (IRES) elements (Belsham & Sonenberg, 2000). Of the proteins identified to date, several appear to be involved in the translation and/or replication of more than one family of viruses. For example, polypyrimidine tract-binding protein (PTB) has been shown to be required for efficient translation from several picornavirus IRES elements (Belsham & Sonenberg, 2000), but also plays a role in Hepatitis C virus IRES-directed translation (Anwar et al., 2000; Gosert et al., 2000) and murine coronavirus replication (Huang & Lai, 1999; Li et al., 1999). In addition to PTB, numerous other RNA-binding proteins, such as unr (Boussadia et al., 2003), poly(rC)-binding protein (PCBP) (Parsley et al., 1997), La (Meerovitch et al., 1993), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Yi et al., 2000), hnRNP A1 (Huang & Lai, 2001) and poly(A)-binding protein (PABP) (Svitkin et al., 2001) have been identified as essential factors for translation or replication of a number of positive-stranded RNA viruses.

Members of the family Caliciviridae of positive-stranded RNA viruses infect a variety of vertebrates. The human caliciviruses (HuCVs) are a major cause of acute gastroenteritis in man and are responsible for a large number of outbreaks in hospitals, cruise ships (Widdowson et al., 2004), military settings (Bailey et al., 2005), nursing homes and restaurants (van Duynhoven et al., 2005). Our understanding of the biology of the HuCVs has been somewhat hindered by the lack of a suitable cell culture system (Duizer et al., 2004), although recent advances have demonstrated limited genome replication and packaging (Asanaka et al., 2005). In contrast, Feline calicivirus (FCV) and the more
recently identified murine norovirus 1 (Karst et al., 2003; Wobus et al., 2004) replicate efficiently in cell culture and represent good models with which to study the general replication and translation strategies employed by caliciviruses. To date, the study of RNA–protein interactions that may play critical roles in calicivirus translation and replication has been limited to in vitro binding studies. For example, work with the HuCV Norwalk virus (NV) has demonstrated that PTB, La, hnRNP L and PCBP-2 interact with 5’ sequences of the positive-sense genomic RNA (Gutierrez-Escolano et al., 2000). PTB and La also interact with the 3’ untranslated region of the NV genome (Gutierrez-Escolano et al., 2003). However, the role of these interactions has yet to be determined due to the lack of a permissive cell line or small animal model to support NV replication.

In the present study we have used FCV as a model system with which to identify and dissect the role of RNA–protein interactions involved in the translation and/or replication of members of the Caliciviridae. We show that PTB interacts specifically with the FCV genomic and subgenomic RNA 5’ sequences. Using RNA interference we further demonstrate that PTB is required for the efficient replication of FCV in cell culture in a temperature-dependent manner. These data are consistent with a role for PTB as an RNA chaperone to stabilize the folding of the viral RNA into a functional state, and this study is the first to present a functional characterization of a role for a host RNA-binding protein in calicivirus replication.

METHODS

Materials. FCV, strain Urbana, was generated by transfection of RNA transcripts derived from the full-length infectious clone pQ14 (Sosnovtsev & Green, 1995) into Crandell–Reese feline kidney (CRFK) cells. Antiserum to the FCV RNA polymerase p76 was generated by immunization of New Zealand White rabbits with recombinant p76 (Sosnovtsev & Green, 1995) into Crandell–Reese feline kidney (CRFK) cells. Antiserum to the FCV RNA polymerase p76 was generated by immunization of New Zealand White rabbits with recombinant p76, purified as described by Wei et al. (2001), from a construct kindly provided by Craig Cameron (Pennsylvania State University, University Park, PA, USA). Antiserum to hnRNP L (PTB) was kindly provided by Richard Jackson (University of Cambridge). The protein was purified from E.coli (packed bed volume) cyanogen bromide-activated Sepharose 4B (Sigma) as described by Kaminski et al. (1995). To isolate proteins that interact specifically with the 5’ sequences of the calicivirus genome and subgenomic RNAs, 50 μl (bed volume) RNA coupled to Sepharose 4B was incubated with 100 μl nuclelease-treated HeLa S10 extract, for 1 h at 4°C. HeLa S10 extracts were prepared as described by Molla et al. (1991). Unbound proteins were removed by extensive washing with S10 buffer (40 mM HEPES, pH 8.0, 120 mM KAc, 5-5 mM MgOAc, 10 mM KCl, 6 mM DTT) and the bound proteins were eluted with 100 μl SDS-PAGE sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to PVDF (Millipore) and immunoblotted for the presence of PTB.

Electrophoretic mobility shift assays (EMSAs). PCR products for the synthesis of radiolabelled FCV 5’-positive-sense genomic and subgenomic RNA probes, encompassing nt 1–245 and 1–284, respectively, as well as unlabelled competitor RNAs, were generated using the primers detailed in Table 1. Labelled and unlabelled transcripts were generated by in vitro transcription, in the presence of [α-32P]GTP where required. All transcripts were purified by electrophoresis on a denaturing urea acrylamide gel, followed by passive elution, as described by Mellits et al. (1990), prior to use in EMSAs. Typically, EMSA reactions contained 85 nM radiolabelled probe, 500 ng GST-PTB, 5 mM HEPES, pH 7.6, 1 mM DTT, 1 mM MgCl2 and 20% glycerol and stored at –80°C until required.

RNA interference-mediated knockdown of PTB. For siRNA-mediated knockdown of PTB expression, CRFK cells were transfected with PTB P1 siRNAs (Domitrovich et al., 2005) (target sequence AACUUCCAUCAUUCCAGAGAA) using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen). Although the sequence of feline PTB is not available, the ability of PTB P1 siRNAs to efficiently lead to a reduction in feline PTB levels would suggest a high degree of sequence conservation. CRFK cells

Table 1. Oligonucleotides used during this study

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IGRDG 13</td>
<td>TAATACGACTCACTATAGGGGTAAAAGAAATTTGAGACAATGTC</td>
</tr>
<tr>
<td>IGRDG 18</td>
<td>GAGATGTTCCACCGTGGAAAG</td>
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<tr>
<td>IGRDG 19</td>
<td>TAAATACGACTCACTATAGGGGTTACTAGGGGTTTTGAGCATG</td>
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were treated in the same way with siRNAs directed towards GFP as a control (Dharmacon). To monitor effective functional PTB knockdown, siRNA-treated CRFK cells were transfected with a poliovirus type 3 luciferase replicon (Goodfellow et al., 2003) that is defective in replication due to a mutation in the 3′ end (mut 4 as described by Meredith et al., 1999) and luciferase levels monitored 16 h post-transfection.

**One-step growth curve.** For the one-step growth curve of FCV at 32 °C, CRFK cells previously treated with PTB or GFP siRNAs were infected at a density of 10⁶ cells per 35 mm dish, with FCV at an m.o.i. of 4 TCID₅₀ per cell. At various times post-infection, virus yield was measured by TCID₅₀ and the levels of the viral polymerase (p76), capsid, PTB and GAPDH were determined by Western blotting for each time point.

For the temperature gradient growth of FCV in cells treated with PTB or GFP siRNAs, 2 x 10⁶ cells were infected in suspension at an m.o.i. of 4 TCID₅₀ per cell. Infections were carried out in an Eppendorf Mastercycler gradient thermal cycler at temperatures between 32 and 39 °C with increments of 1 °C (±0.1 °C). To maintain the correct pH, the culture medium was supplemented with 25 mM HEPES, pH 7.0. Cells were lysed at 6 h post-infection and analysed by Western blotting with antisera to FCV p76 to monitor viral protein synthesis, PTB and GAPDH.

**Northern blot analysis.** RNA was extracted from cells at various times post-infection using the Genelute purification system (Sigma). RNA (1 μg) was treated with glyoxal for 30 min at 65 °C (Ambion) prior to separation on a 0.8% agarose gel. RNA was transferred to nitrocellulose (Amersham Biosciences) under mild alkaline conditions by capillary transfer (Sambrook & Russell, 2001). RNA was detected using an antisense RNA probe consisting of nt 5297–7683, encompassing the entire subgenomic RNA.

**RESULTS**

**PTB binds to the 5′ sequences of the FCV genomic and subgenomic RNAs**

To identify cellular RNA-binding proteins which play a role in the calicivirus life cycle, affinity columns containing the 5′ sequences of the FCV genomic and subgenomic RNAs were generated by coupling in vitro-transcribed RNAs to cyanogen bromide-activated Sepharose 4B. In the absence of any functional data with regard to the position of RNA sequences required for genome translation and/or replication, we chose to use the 5′ extremities of both the genomic and subgenomic RNAs (nt 1–245 and 1–284, respectively) as these regions are predicted to contain significant levels of RNA structure (data not shown). Cytoplasmic extracts from HeLa cells were used for affinity purification of host-cell factors that specifically interact with the RNA elements from the two regions. HeLa cells were chosen as a source of protein as, although not permissive for FCV infection, high levels of virus can be recovered after transfection of VPg-linked FCV RNA (data not shown). Hence, all the necessary factors for efficient genome translation and replication were present and the inability to support FCV infection would be likely to reflect a lack of a suitable cellular receptor or a defect in virus entry.

We initially chose to examine the presence of PTB in eluates from the RNA affinity columns as PTB has previously been shown to bind the 5′ and 3′ extremities of the NV genome (Gutierrez-Escolano et al., 2000, 2003). In addition, the presence of PTB-binding site consensus sequences (UCUU (Singh et al., 1995) within the 5′ extremities of both genomic and subgenomic RNAs (data not shown) further suggested this protein may bind to this region. In agreement with this, PTB was detected in the eluates from the 5′ genomic and 5′ subgenomic RNA affinity columns (Fig. 1a), whereas only background levels of PTB were recovered from columns containing the E. coli 55 rRNA or columns lacking RNA (Fig. 1a).

**PTB interacts directly with the 5′ extremities of the FCV genomic and subgenomic RNAs**

To confirm that PTB interacts directly with the FCV RNA, rather than an indirect interaction via an additional cellular factor, EMSAs were performed using recombinant GST-PTB. GST-PTB was found to interact with RNA probes encompassing nt 1–245 and 1–284 of the FCV genomic and subgenomic RNAs, respectively (Fig. 1b, c). GST alone was found not to interact with either of the target RNAs (data not shown). A complex with apparent reduced mobility, present in the absence of recombinant PTB (highlighted with an asterisk in Fig. 1b), is likely to represent dimeric probe as it could be resolved by annealing of the RNA probe prior to use (data not shown).

To determine the specificity of the interaction, the effect of adding an excess of unlabelled RNA competitors to EMSA reactions was examined. A non-specific RNA consisting of the poliovirus 2C cis-acting replication element (Goodfellow et al., 2000) did not inhibit the interaction of PTB with the genomic or subgenomic RNA probes (Fig. 1b, c). In addition, the 3′ sequences of the FCV genome (nt 7574–7683) had no effect on the PTB-5′ end complex (data not shown). Homologous unlabelled FCV genomic and subgenomic RNA 5′ extremities were found to inhibit complex formation efficiently, confirming a specific interaction between PTB and the FCV genomic and subgenomic RNA extremities (Fig. 1b, c).

**PTB is required for the efficient replication of caliciviruses in a temperature-dependent manner**

To analyse a role for PTB in the FCV life cycle, CRFK cells were transfected with siRNAs directed towards PTB, and the levels of PTB were monitored by Western blot analysis. Transfection of PTB-specific siRNAs resulted in an 80% decrease in PTB levels compared to cells transfected with siRNAs directed towards GFP (Fig. 2). To confirm a ‘functional effect’ of the reduced levels of PTB in CRFK cells, the effect of PTB siRNAs on poliovirus IRES-mediated translation was examined. A poliovirus replicon, defective in replication due to a mutation in the 3′ untranslated region (mut 4 as described in Meredith et al., 1999) was transfected into CRFK cells previously treated with PTB- or GFP-specific siRNAs. PTB knockdown resulted in a greater than
200-fold decrease in poliovirus IRES-directed expression of luciferase (0.081 vs 17.5 relative light units for PTB- and GFP-specific siRNAs, respectively), in agreement with previous work (Florez et al., 2005).

To examine the functional role of PTB in the FCV life cycle, the effect of PTB knockdown on FCV polymerase (p76) production was examined over a range of temperatures by Western blot analysis and quantified by densitometry (Fig. 3). PTB siRNAs were found to have a greater effect on p76 levels at temperatures above and below 37°C. Whereas at 37°C p76 levels were 65% of that observed in GFP siRNA transfected cells, at 32 and 39°C the levels were reduced to 20 and 18%, respectively (Fig. 3).

The effect of PTB knockdown on FCV replication at 32°C was examined in more detail by analysing virus yield, RNA synthesis and the levels of viral protein (Fig. 4). This temperature was chosen for more detailed analysis as at temperatures above 37°C, the replication and yield of FCV (strain Urbana) was significantly reduced (data not shown). At 32°C however, although replication occurred at a reduced rate, similar levels of virus and viral proteins were produced. PTB knockdown was found to have a dramatic effect on FCV yield at 32°C, resulting in a 100-fold reduction in titre at 6 h post-infection, with a 21- and 15-fold reduction seen at 9 and 18 h, respectively (Fig. 4a).

Similarly, the appearance of cytopathic effect was inhibited, with a proportion of cells remaining intact after 18 h of infection in PTB siRNA-treated cells, whereas infection of GFP siRNA-transfected cells resulted in a typical virus induced cytopathic effect (Fig. 4b).

The viral polymerase (p76) and major capsid protein were quantified as a measure of the levels of translation from the genomic and subgenomic RNAs, respectively (Fig. 4c). Levels of p76 and capsid were reduced by similar levels in
PTB siRNA-transfected cells, resulting in a sixfold reduction in protein synthesis at 6 h post-infection.

The levels of the FCV genomic and subgenomic RNAs were examined by Northern blotting and, as observed for viral protein production, levels were reduced in PTB siRNA-treated cells (Fig. 4d, e). Genomic and subgenomic RNA production was inhibited to the same degree (data not shown), indicating that PTB knockdown did not have a differential effect on subgenomic RNA synthesis. Genomic RNA levels were 42, 56 and 75% of the levels observed in GFP siRNA-treated cells at 6, 9 and 18 h post-infection, respectively (Fig. 4d, e).

**DISCUSSION**

In general, the 5' and 3' extremities of viral RNA genomes must fold into defined three-dimensional structures in order to adopt a functional state. As a result of structural promiscuity and an abundance of intramolecular interactions, alternative non-functional conformations are also adopted, significantly slowing the appearance of a functional conformation (Treiber & Williamson, 2001). As a consequence, it has been suggested that RNA folding requires the aid of proteins with chaperone activity that possibly trap or resolve misfolded structures (Herschlag, 1995).

The interaction of host-cell nucleic acid-binding proteins with the genomes of positive-stranded RNA viruses is known to play a role in many aspects of the virus life cycle. The majority of these proteins are predicted to function as RNA chaperones, allowing the viral RNA to adopt a functional conformation. In the case of cellular or viral IRES elements, the binding of host-cell proteins to RNA is thought to lead to structural rearrangements, usually in close proximity to the protein-binding site, resulting in the formation of a conformation suitable for translation initiation (Martínez-Salas et al., 2001). These proteins, known as IRES trans-acting factors (ITAFs), include PTB isoforms, poly(rC)-binding protein, La, hnRNP K, unr (upstream of N-ras), nucleolin and many others (reviewed by Stoneley & Willis, 2004).

Here we report the identification of PTB as a host-cell protein required for efficient calicivirus replication in a temperature-dependent manner. PTB is a regulator of alternative splicing pathways (Lin & Patton, 1995) and is predominantly found in the nucleus, although it can shuttle between the nucleus and cytoplasm (Ghetti et al., 1992).
Recent work has demonstrated that phosphorylation of PTB by protein kinase A results in the accumulation of PTB in the cytoplasm (Xie et al., 2003). PTB was found to be evenly distributed between the nucleus and the cytoplasm during poliovirus infection, probably as a result of the inhibition of host-cell transcription and the effects of virus infection on nuclear import (Back et al., 2002). Cleavage of PTB has also been observed during poliovirus infection with the resultant cleavage products inhibiting viral translation (Back et al., 2002). This inhibition is thought to contribute to the switch from translation to replication (Back et al., 2002). Whether a similar cleavage and redistribution of PTB occurs during FCV infection is currently being determined.

A more important role for PTB at temperatures above and below 37 °C would agree with the hypothesis that PTB functions as an RNA chaperone, aiding in the correct folding of viral RNA. The lack of a significant effect of PTB siRNAs on FCV replication at 37 °C, the temperature at which the virus has been repeatedly passaged, is intriguing. Although viral RNA polymerase levels were reduced to 65% of the levels observed in control siRNA-treated cells (Fig. 3), no
effect on virus titre was observed (data not shown). It is possible that the RNA chaperone activity of PTB is only required in conditions where non-functional RNA structures are stabilized (e.g. temperatures <37°C) or functional structures are destabilized (e.g. temperatures >37°C). It is important to note that although RNA interference-mediated knockdown of PTB significantly reduced PTB levels, detectable PTB remained (Fig. 2). Hence, the PTB remaining after siRNA-mediated knockdown may be sufficient for correct RNA folding at 37°C, but increased levels are required to maintain a functional conformation at non-favourable temperatures or in circumstances where other RNA chaperone factors are absent. Previous studies on EMCV IRES-mediated translation have demonstrated that although a wild-type IRES directing EMCV polyprotein synthesis does not require PTB for efficient translation, an IRES with an enlarged A-rich bulge is highly dependent on PTB (Kaminski & Jackson, 1998). As a result, it was presumed that PTB plays a significant role in maintaining an appropriate higher order structure for translation initiation only when non-functional conformations are apparent (Kaminski & Jackson, 1998). This observation would fit with our hypothesis that the function of PTB in the calicivirus life cycle is only required under 'unfavourable' conditions.

An additional factor that may affect the relative requirement of RNA for a particular RNA chaperone is the expression levels of other interacting host-cell factors. The relative expression levels of such factors may be significantly different in primary tissues compared to the levels observed in immortalized cell lines. The temperature of the environment in which the virus replicates is also likely to be a determining factor in the relative contribution of PTB to the virus life cycle. Given that feline body core temperature ranges from 38 to 39°C and can rise to 41.5°C during FCV infection (Poulet et al., 2005), we would predict PTB plays a functional role in FCV replication in vivo.

Similar temperature sensitivity of the RNA chaperone activity of PTB has also been highlighted during trans-splicing of the thymidylate synthase group 1 intron (Belisova et al., 2005). Whereas trans-splicing occurs in a protein-independent manner at 55°C, the reaction is significantly reduced at 37°C due to an inability of the RNA to fold into a splicing-competent conformation. PTB was found to stimulate the rate of trans-splicing at 37°C by threefold (Belisova et al., 2005). This increased requirement for PTB at reduced temperature was due to stabilization of RNA structures that were non-functional for splicing.

Previous data on the interaction of PTB with the NV genome demonstrated that PTB interacts with both the 5’ and 3’ extremities of the viral genomic RNA (Gutierrez- Escolano et al., 2000, 2003). However, in the current study we failed to detect a PTB–3’-end interaction by both UV cross-linking and EMSA (data not shown). This may suggest that the caliciviruses differ in their requirements for host-cell RNA-binding proteins.

We have recently reported that caliciviruses use a novel translation initiation mechanism not seen in any other animal RNA viruses, whereby a protein covalently linked to the 5’ end of viral RNA (VPg) functions as a proteinaceous cap substitute, recruiting components of the eIF4F complex (Goodfellow et al., 2005). Previous work has also demonstrated an interaction of norovirus VPg with eIF3 (Dauganbaugh et al., 2003), although a role for this interaction in calicivirus translation has yet to be determined. Given the previous reports of a role for PTB in viral translation (reviewed by Stoneley & Willis, 2004), it is possible that PTB also plays a role in calicivirus translation. From the current study we are unable to determine the specific role of PTB in the calicivirus life cycle. This is primarily due to a lack of a specific inhibitor that prevents RNA replication but allows translation to occur, analogous to the effect of guanidine hydrochloride on poliovirus (Richtsel et al., 1961). Preliminary results with rabbit reticulocyte lysates depleted of PTB would suggest that PTB does not play a significant role in calicivirus VPg-dependent translation (data not shown). However, confirmation of this awaits the development of a reproducible method of specifically depleting PTB from translation-competent extracts prepared from permissive cells, as rabbit reticulocyte lysates may not faithfully reproduce the effect of PTB depletion on translation alone, due to the increased levels of translation initiation factors and the lack of compartmentalization. Although a previous study has highlighted that the primary role for PTB in the life cycle of many positive-stranded RNA viruses is at the level of viral translation (Belsham & Sonenberg, 2000); a role in coronavirus replication has also been observed (Huang & Lai, 1999; Li et al., 1999). Previous work has also shown that PTB binds to 3’ sequences (the ‘X’ sequence) in the HCV genome and may play a role in HCV replication (Domitrovich et al., 2005; Gontarek et al., 1999). Hence, it is possible that PTB plays no role in calicivirus translation, but instead is required for some aspect of viral RNA replication.

FCV infection generally results in an oral or upper respiratory tract infection (Gaskell et al., 2004); however, recent isolates can result in a highly contagious febrile haemorrhagic syndrome (Hurley & Sykes, 2003). FCV vaccines, based on live attenuated or inactivated preparations are available, but whilst they are effective at preventing disease, they do not prevent infection. Antigenic variation has resulted in new strains for which vaccines do not offer protection and there are also concerns that the currently available vaccines may contribute to FCV prevalence (Radford et al., 2001). Hence the current study, in addition to yielding insights into the general biology of the calicivirus life cycle, may allow the rational design of attenuated FCV vaccines, as mutations in PTB-binding sites have been shown to contribute to poliovirus attenuation (Gutierrez et al., 1997).

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