Caliciviruses Differ in Their Functional Requirements for eIF4F Components*  

Received for publication, March 9, 2006, and in revised form, June 7, 2006  
Published, JBC Papers in Press, July 11, 2006, DOI 10.1074/jbc.M602230200

Yasmin Chaudhry‡, Arabinda Nayak§, Marie-Eve Bordeleau‡, Junichi Tanaka¶, Jerry Pelletier**2, Graham J. Belshaw§¶, Lisa O. Roberts**3, and Ian G. Goodfellow‡5  

From the ‡Department of Virology, Faculty of Medicine, Imperial College London, St. Mary’s Campus, Norfolk Place, London W2 1PG, United Kingdom, the §Biotechnology and Biological Sciences Research Council Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, United Kingdom, the ¶Department of Biochemistry, McIntyre Medical Sciences Building, McGill University, Montreal, Quebec H3G 1Y6, Canada, the **Department of Chemistry, Biology, and Marine Sciences, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan, and the ***School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom

Two classes of viruses, namely members of the Potyviridae and Caliciviridae, use a novel mechanism for the initiation of protein synthesis that involves the interaction of translation initiation factors with a viral protein covalently linked to the viral RNA, known as VPg. The calicivirus VPg proteins can interact directly with the initiation factors eIF4E and eIF3. Translation initiation on feline calicivirus (FCV) RNA requires eIF4E because it is inhibited by recombinant 4E-BP1. However, to date, there have been no functional studies carried out with respect to norovirus translation initiation, because of a lack of a suitable source of VPg-linked viral RNA. We have now used the recently identified murine norovirus (MNV) as a model system for norovirus translation and have extended our previous studies with FCV RNA to examine the role of the other eIF4F components in translation initiation. We now demonstrate that, as with FCV, MNV VPg interacts directly with eIF4E, although, unlike FCV RNA, translation of MNV RNA is not sensitive to 4E-BP1, eIF4E depletion, or foot-and-mouth disease virus Lb protease-mediated cleavage of eIF4G. We also demonstrate that both FCV and MNV RNA translation require the RNA helicase component of the eIF4F complex, namely eIF4A, because translation was sensitive (albeit to different degrees) to a dominant negative form and to a small molecule inhibitor of eIF4A (hippuristanol). These results suggest that calicivirus RNAs differ with respect to their requirements for the components of the eIF4F translation initiation complex.

Translation initiation on eukaryotic mRNAs is a complex process, and many translational control mechanisms are focused on the initiation stage (1–3). The majority of host cell mRNAs are translated in a cap-dependent manner involving the recognition of their 5′ cap structure by the eIF4F initiation factor complex (4). eIF4F is known as the “cap-binding complex” and comprises three proteins: (i) eIF4E, the only factor with direct cap-binding activity; (ii) eIF4A, an RNA helicase; and (iii) eIF4G, which functions as a scaffold to bind several other factors such as eIF3, poly(A)-binding protein (PABP),6 eIF4E and eIF4A. Subsequent to eIF4F binding to the 5′ cap, the 43 S preinitiation complex is recruited to the mRNA via its interaction with eIF3 (5).

Positive-stranded RNA viruses have evolved a variety of mechanisms for subverting the host cell translation machinery for their own use (6, 7). In many cases this results in the preferential translation of viral mRNAs in the presence of relatively high concentrations of competing host cell mRNAs. However, infection of cells by many picornaviruses also leads to the inhibition of host cell (cap-dependent) translation. This is primarily achieved through the cleavage of eIF4G, for example by the poliovirus 2A protease (8, 9) or foot-and-mouth disease virus L protease (10), resulting in the separation of the eIF4A- and eIF4E-binding sites. Picornavirus mRNAs are still translated because of the presence of an internal ribosome entry site (IRES) element in the 5′-untranslated region that directs a cap-independent mechanism of translation. The C-terminal cleavage fragment of eIF4G is generally sufficient to support picornavirus IRES function (6).

Caliciviruses are a major cause of viral gastroenteritis and have been associated with over 85% of nonbacterial gastroenteritis outbreaks in Europe between 1995 and 2000 (11). The human caliciviruses, including the prototype Norwalk virus, have yet to be fully propagated in tissue culture, although recent results suggest that limited genome replication and encapsidation can occur using a vaccinia virus-driven expression system (12). In contrast to the human caliciviruses, feline calicivirus (FCV), porcine enteric calicivirus (13, 14), and murine norovirus 1 (MNV) (15) can be propagated in tissue culture. Reverse genetics systems also exist for both FCV (16) and porcine enteric calicivirus (17). Therefore FCV,

---

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1Present address: Dept. of Microbiology and Immunology, University of California, San Francisco, CA 94143-2280.

2Supported by the National Cancer Institute of Canada Grant 014313.

3Supported by the Biotechnology and Biological Sciences Research Council (BBSRC).

4Present address: Danish Institute for Food and Veterinary Research, Dept. of Virology, Lindholm, DK-4771 Kalvehave, Denmark.

5Supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council (BBSRC). To whom correspondence should be addressed. Tel.: 44-20-7594-2002; Fax: 44-20-7594-3973; E-mail: l.Goodfellow@imperial.ac.uk.

6The abbreviations used are: PABP, poly(A)-binding protein; FCV, feline calicivirus; MNV, murine norovirus; IRES, internal ribosome entry site; LDV, Lordsdale virus; CRFK, Crandell-Rees feline kidney; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; FMDV, foot and mouth disease virus.
porcine enteric calicivirus, and MNV have been used as model systems to study calicivirus biology.

We and others have previously reported that caliciviruses use a novel protein-directed translation initiation mechanism that involves the binding of translation initiation factors to the VPg protein that is covalently linked to the 5′ end of the viral RNA (18, 19). This mechanism has not been demonstrated in any other animal RNA virus but shares some similarity with a mechanism proposed for members of the plant potyvirus family (20–23). In our previous studies, we demonstrated that the VPg proteins of both FCV and Lordsdale virus (LDV), a human norovirus, interact directly with the eIF4E component of the eIF4F complex (19). Translation of FCV VPg-linked mRNA was blocked by the eIF4E inhibitor protein, 4E-BP1, confirming a functional role for eIF4E and suggesting that the eIF4E-4G interaction is essential for FCV translation. Because of the lack of functional role for eIF4E and 4G, MNV VPg-linked RNA was prepared from RAW 264.7 cells, infected at a multiplicity of infection of 2 TCID50/cell, at 18 h post-infection, using the same system.

Isolation of Calicivirus VPg-linked RNA from Infected Cells—FCV VPg-linked RNA was prepared from replication complexes isolated 4 h post-infection using the Genelute purification system (Sigma) as previously described (19). MNV VPg-linked RNA was prepared from RAW 264.7 cells, infected at a multiplicity of infection of 2 TCID50/cell, at 18 h post-infection, using the same system.

Detection of VPg in MNV VPg-linked RNA Preparations—RNA isolated from either mock infected or MNV-infected cells was digested with RNase mixture (Ambion) for 1 h at 37 °C prior to analysis by Western blotting with affinity purified anti-MNV VPg antiserum.

In Vitro Translation Reactions—In vitro translation reactions were performed using the Flexi rabbit reticulocyte lysate system (Promega), using 25, 50, and 12.5 μg/ml of FCV, MNV, and control in vitro transcribed RNAs, respectively. These concentrations of RNA were previously determined to give a linear yield of translated product over the time course of the translation (90 min). In reactions that required the addition of either recombinant 4E-BP1, eIF4E, or the dominant negative mutant (DQAD) form of eIF4A, the reactions were preincubated with recombinant protein at 30 °C for 15 min prior to the addition of RNA. After 90 min, the reactions were terminated by the addition of SDS-PAGE sample buffer and subsequently resolved on 12.5% polyacrylamide gels. Pretreatment of RNA with proteinase K was carried out by incubation of RNA in 10 mM Tris, pH 8.0, 0.1 mM EDTA, 10 μg/ml proteinase K for 30 min at 37 °C followed by extraction with phenol:chloroform and precipitation with ethanol. MNV RNA was also treated as above but with the omission of proteinase K or the addition of EDTA-free protease inhibitor mixture (Roche Applied Science) as additional controls. Control in vitro transcribed RNA (of the form cap-CAT:IRES-Luc) (28) was treated in the same manner. In reactions that contained exogenous cap analogue, 50 or 500 μM cap analogue (m7G(5′)ppp(5′)G, Promega) was preincubated with reticulocyte lysate at 30 °C for 15 min prior to the addition of RNA template.

eIF4E Depletion—eIF4E was depleted from RRL as previously described (29). Briefly, 4E-BP1 (400 nM) was incubated with RRL at 30 °C for 10 min prior to the addition of 0.5 volume of cap-Sepharose. Bound complexes were removed by centrifugation, and the depleted lysate was aliquoted and frozen at −80 °C until required.

In Vitro Transcription—Capped dicistronic mRNA was prepared from XhoI linearized pGEM-CAT:IRES-Luc (28) or pGEM-rLuc:IRES-Luc in which the CAT coding region was replaced with Renilla luciferase, using the Megascript transcription system (Ambion) in the presence of cap analogue (Promega). RNA was purified by lithium chloride precipitation and quantified by spectrophotometry.

eIF4E Capture ELISA—Capture ELISAs were used to detect the interaction of eIF4E with VPg were performed essentially as described previously (22) except that murine eIF4E-GST or
GST alone were purified using glutathione-Sepharose chromatography (GE Healthcare). The interaction of initiation factors with VPg was detected using an anti-GST monoclonal antibody.

**Cap-Sepharose Chromatography**—Lysates from uninfected or infected RAW 264.7 cells, prepared 18 h post-infection with MNV, were incubated with cap-Sepharose (GE Healthcare) and eIF4E-containing complexes isolated as previously described (30). Bound proteins were eluted with SDS-PAGE sample buffer and analyzed by Western blot.

**Virus Yield Assays**—CRFK or RAW 264.7 cells were pre-treated with hippuristanol (125 nM to 1 μM) or Me₂SO as a control, for 1 h prior to infection with FCV or MNV (multiplicity of infection of 2). Infections were carried out in the presence of inhibitor/Me₂SO for 30 min at room temperature, after which virus and inhibitor were removed, and the cells were washed with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells were then incubated in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum containing hippuristanol or Me₂SO at 37 °C for 6 or 18 h for FCV and MNV, respectively. After two freeze/thaw cycles, the virus yield was determined by TCID50 on CRFK and RAW 264.7 cells for FCV and MNV, respectively.

**Cell Viability Assay**—The effect of hippuristanol on the viability of CRFK and RAW 264.7 cells was examined using the CellTitre-Blue system (Promega) according to the manufacturer’s instructions. CRFK and RAW 264.7 cells were incubated with various concentrations of inhibitor or Me₂SO for 4 and 16 h, respectively. CellTitre-Blue reagent was then added to each well, and incubation continued for a further 2 h, after which the level of fluorescence was determined according to the manufacturer’s instructions.

**RESULTS**

**Murine Norovirus RNA Translation Is Insensitive to Cap Analogue**—To date, the study of norovirus translation has been limited to in vitro binding analysis of the recombinant norovirus VPg with translation initiation factors (18). To fully evaluate the functional roles of initiation factors in norovirus translation, an in vitro translation system was required. MNV has previously been shown to replicate efficiently in the STAT1-negative murine macrophage cell line RAW 264.7 (15), hence this offered a potential source of norovirus VPg with translation initiation factors (18).

MNV has been shown to replicate efficiently in the STAT1-negative murine macrophage cell line RAW 264.7 (15), hence this offered a potential source of norovirus VPg with translation initiation factors (18). To fully evaluate the functional roles of initiation factors in norovirus translation, an in vitro translation system was required. MNV has previously been shown to replicate efficiently in the STAT1-negative murine macrophage cell line RAW 264.7 (15), hence this offered a potential source of norovirus VPg-linked RNA. The MNV VPg-linked RNA was prepared by extracting total RNA from RAW 264.7 cells at various times post-infection. RNA prepared in a similar manner to FCV-infected cells was previously found to translate efficiently in the rabbit reticulocyte lysate system (19). Using similar in vitro translation conditions, RNA prepared from MNV-infected cells was translated efficiently in rabbit reticulocyte lysates (Fig. 1A). The translation profile of the...
RNA isolated at 6 or 18 h post-infection varied (Fig. 1A). Translation of a host cell mRNA present in uninfected extracts, highlighted in Fig. 1A with an asterisk, diminished during the course of infection and was absent when RNA was prepared from cells 18 h post-infection. However, the synthesis of some virus encoded products (e.g. 32-kDa product) was greatly enhanced using the RNA isolated at 18 h post-infection compared with the RNA isolated at 6 h.

As we previously observed with FCV VPg-linked RNA, translation of MNV mRNA was found to require a protein covalently linked to the viral RNA (VPg), because pretreatment of the RNA with proteinase K ablated translation (Fig. 1B). The inclusion of protease inhibitors in the reaction prevented the effect of proteinase K (Fig. 1B). In contrast, translation of in vitro synthesized, control capped dicistronic RNA was unaffected by proteinase K pretreatment (Fig. 1B).

We and others have previously demonstrated that FCV translation was insensitive to the addition of exogenous cap analogue (m7G(5'ppp(5')G))(19, 31). To determine whether MNV mRNA translation also occurred in a cap analogue-insensitive manner, the effect of cap analogue on MNV mRNA in vitro translation was examined. Cap analogue had no significant effect on translation of MNV VPg-linked mRNA (Fig. 1C). Cap-dependent translation from in vitro synthesized control dicistronic was unaffected (Fig. 1D).

Further evidence that the observed in vitro translation profile was the result of VPg-dependent translation was the observation that RNA prepared in this manner was found to be infectious after transfection into permissive cells but noninfectious after treatment with proteinase K (data not shown).

MNV VPg Interacts with eIF4E—We have previously demonstrated a direct interaction of the FCV and LDV VPg proteins with eIF4E in vitro (19). This interaction was also observed in infected cells because FCV VPg could be isolated using a cap-Sepharose affinity resin. To determine whether a similar interaction existed between MNV VPg and eIF4E, recombinant MNV VPg was expressed and purified from E. coli as a C-terminal fusion to a six-histidine tag (A). The interaction of murine eIF4E with either FCV or MNV VPg was assayed by capture ELISA. Wells were precoated with 10 μg of FCV or MNV VPg, maltose-binding protein (MBP) or 4E-BP1 and incubated with 5 μg of GST-eIF4E or GST alone. Complexes were detected using anti-GST antibodies, and the optical density at 420 nm was quantified (B). Extracts from either mock infected (M) and MNV infected (MN) cells were incubated with either Sepharose 4B or cap-Sepharose as described (30), and bound proteins were analyzed by Western blotting for both VPg, elf4A, elf4E, elf4G, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, C). RNA isolated from mock infected (M) or MNV-infected (MN) cells was digested with RNases and analyzed for the presence of VPg by Western blot (D).

FIGURE 2. Murine norovirus VPg interacts with elf4E in vitro and in vivo. Murine norovirus VPg was expressed and purified from E. coli as a C-terminal fusion to a six-histidine tag (A). The interaction of murine eIF4E with either FCV or MNV VPg was assayed by capture ELISA. Wells were precoated with 10 μg of FCV or MNV VPg, maltose-binding protein (MBP) or 4E-BP1 and incubated with 5 μg of GST-eIF4E or GST alone. Complexes were detected using anti-GST antibodies, and the optical density at 420 nm was quantified (B). Extracts from either mock infected (M) and MNV infected (MN) cells were incubated with either Sepharose 4B or cap-Sepharose as described (30), and bound proteins were analyzed by Western blotting for both VPg, elf4A, elf4E, elf4G, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, C). RNA isolated from mock infected (M) or MNV-infected (MN) cells was digested with RNases and analyzed for the presence of VPg by Western blot (D).
all were retained on cap-Sepharose; however, the mature form and the ~32-kDa precursor appear to be enriched (Fig. 2C). The levels of the eIF4F components present in cells or isolated by cap-Sepharose chromatography were unaffected by infection (Fig. 2C). Mature VPg, VPg precursors, or eIF4F components were not isolated on control Sepharose 4B alone (Fig. 2C). Glyceraldehyde-3-phosphate dehydrogenase was also not retained by cap-Sepharose, confirming the specificity of the assay (Fig. 2C).

Only the Mature Form of VPg Is Linked to Viral RNA—Given our observation that all forms of VPg can be isolated by cap-Sepharose chromatography, we wished to examine the form of VPg found covalently linked to viral RNA. RNA isolated from either mock infected or MNV-infected cells was digested with RNase and subsequently analyzed by Western blot. In agreement with previous findings with FCV (31), only the mature form of VPg was found to be covalently linked to MNV RNA (Fig. 2D).

Translation of MNV RNA Is Not Inhibited by 4E-BP1—Our previous analysis revealed that in vitro translation of VPg-linked FCV mRNA was sensitive to the addition of the eIF4E repressor protein 4E-BP1 (19). This protein binds to eIF4E and prevents the interaction with eIF4G (4). These data would therefore suggest that the eIF4E-4G interaction is required for FCV mRNA translation. To determine whether a similar interaction is required for MNV mRNA translation, the effect of recombinant 4E-BP1 was examined (Fig. 3, A and B). Whereas recombinant 4E-BP1 inhibited FCV and cap-dependent translation from a control dicistronic mRNA as expected, MNV mRNA translation and FMDV IRES-directed translation were unaffected (Fig. 3, A and B). A protein translated from the MNV VPg-linked RNA preparations was also found to be sensitive to 4E-BP1 addition (Fig. 3A, asterisk). This is likely to represent a host cell mRNA that is translated in a cap-dependent manner and, where visible, will be referred to as CPX.

Depletion of eIF4E Differentially Affects Calicivirus Translation—To further determine whether the MNV VPg-eIF4E interaction plays a significant role in MNV translation initiation, the translation of calicivirus RNA was examined in
**Calicivirus Translation Initiation**

RRL depleted of eIF4E (Fig. 3C), eIF4E was depleted as described (29), and the concomitant removal of eIF4G was prevented by the prior addition of 4E-BP1. The eIF4E:eIF4G-BP1 was subsequently removed by cap-Sepharose. Western blot analysis of depleted lysates demonstrated that whereas eIF4E levels were typically less than 10% of the levels seen in mock depleted lysates, the levels of the other eIF4F components, eIF4A and eIF4G, remained largely unaltered (Fig. 3D). Analysis of the levels of recombinant 4E-BP1 remaining in the depleted extract revealed that greater than 90% of the 4E-BP1 added to the reaction was removed during depletion (Fig. 3D). Note that the rabbit polyclonal antiserum used to detect the recombinant 4E-BP1 did not detect the endogenous 4E-BP1 present in the RRL (Fig. 3D). eIF4E depletion was found to specifically inhibit cap and FCV VPg-dependent translation, whereas FMDV IRES-mediated translation was largely unaffected (Fig. 3C). The addition of recombinant His-tagged eIF4E to a final concentration of ~0.6 μM restored cap and FCV VPg-dependent translation to the levels found in mock depleted lysates (Fig. 3C). However, the addition of recombinant eIF4E to the depleted lysates did not restore MNV translation, suggesting that the observed effect was not due to the removal of eIF4E.

**FCV and MNV RNAs Differ in Their Requirements for Intact Full-length eIF4G**—eIF4G plays several critical roles in translation initiation and is recruited to the 5’ end of mRNA via its interaction with eIF4E (32). To confirm that the eIF4E-eIF4G interaction was not required for MNV translation, the eIF4E-interacting domain of eIF4G was separated from the C terminus of eIF4G by cleavage with FMDV Lb protease, and the effect on calicivirus translation was analyzed (Fig. 4). Prior incubation of rabbit reticulocyte lysates with recombinant Lb protease led to a dose-dependent cleavage of eIF4G (Fig. 4A). As expected, eIF4G cleavage resulted in inhibition of cap-dependent translation, whereas FMDV IRES-directed translation was unaffected (Fig. 4, B and E). eIF4G cleavage resulted in inhibition of FCV mRNA translation but resulted in a slight stimulation of MNV translation (Fig. 4, C–E) probably because of a decrease in competition from cellular mRNAs or release of eIF4G from the eIF4E-eIF4G complex. A protein translated from MNV RNA preparations, described above as CPX (Fig. 4D, asterisk), was inhibited in reactions where eIF4G was cleaved with Lb protease (Fig. 4, D and E).

Both MNV and FCV RNAs Require eIF4A for Translation Initiation—A role for the remaining eIF4F component, namely the RNA helicase eIF4A, was initially examined by studying the effect of a recombinant dominant negative form of eIF4A on translation. The dominant negative mutant form of eIF4A containing a mutation in the ATPase B motif that changes the sequence DEAD to DQAD, herein referred to as eIF4ADQAD, results in inactivation of the ATPase and helicase activity (26). The eIF4ADQAD was found to inhibit cap-dependent translation as expected (Fig. 5A). As previously reported, translation from the porcine teschovirus (PTV-1) IRES was unaffected by this inhibitor (Fig. 5A) (27). MNV RNA translation was found to be highly sensitive to inhibition by eIF4ADQAD, with 1 μg resulting in almost complete inhibition (Fig. 5C). FCV RNA translation was also sensitive to eIF4ADQAD; however, higher concentrations were required to inhibit efficiently. Using 1 μg of the inhibitor protein reduced FCV RNA translation by ~75% and even in the presence of 2 μg of eIF4ADQAD, translation still occurred (Fig. 5B).

**A Small Molecule Inhibitor of eIF4A, Hippuristanol, Inhibits Translation of Both FCV and MNV RNAs in Vitro**—To confirm that eIF4A was required for both MNV and FCV translation, the effect of a small molecule inhibitor of eIF4A...
function, hippuristanol, was examined. The inhibitor is a sterol isolated from the coral *Isis hippuris* and was identified via a high throughput screen for general translation inhibitors (25). Hippuristanol has been shown to function by binding to the C-terminal domain of eIF4A, inhibiting the RNA binding, ATPase activity, and helicase activities of eIF4A (25). As expected, hippuristanol was found to inhibit cap-dependent translation in a dose-dependent manner, whereas translation from the PTV-1 IRES was unaffected (Fig. 6A).

Translation of both FCV and MNV mRNA was inhibited by hippuristanol, although to different levels (Fig. 6, B–D). Complete inhibition of FCV RNA translation was observed at 1 μM hippuristanol, whereas complete inhibition of MNV RNA translation was observed at the 5-fold lower concentration of 0.2 μM.

**Hippuristanol Inhibits FCV and MNV Virus Production**—In addition to functioning as an efficient *in vitro* inhibitor of eIF4A function, hippuristanol has also been demonstrated to be cell-permeable and efficiently inhibit eIF4A function *in vivo* (25). Hence, the effect of hippuristanol on the replication of both FCV and MNV was examined (Fig. 7A). As observed with the *in vitro* studies, MNV was found to be significantly more sensitive to hippuristanol than FCV. MNV virus yield was decreased by over 3000-fold at 250 nM hippuristanol, whereas FCV yield was unaffected (Fig. 7A). At 1 μM hippuristanol the yield of FCV was reduced by over 170,000-fold. Previous studies with hippuristanol have highlighted its cytotoxic nature, most likely because of inhibition of host cell cap-dependent translation (25). To control for the effects of hippuristanol on virus yield caused by a significant decrease in cell viability, the cytotoxicity of hippuristanol was assayed. The viability of CRFK and RAW 264.7 cells was examined after exposure for 6 and 18 h, respectively (Fig. 7B). CRFK cells were largely unaffected by treatment with hippuristanol for 6 h, the time at which maximal virus yield is obtained from a single growth cycle of FCV (Fig. 7B). In contrast, exposure of RAW 264.7 cells to hippuristanol for 18 h, the time required for maximal virus yield resulted in a marked decrease in cell viability at concentrations greater than 250 nM (Fig. 7B). It is important to note, however, that at a hippuristanol concentration of 250 nM, 90% of cells retained viability yet MNV yield was reduced by over 3000-fold (highlighted in Fig. 7), suggesting that this inhibitor specifically affects MNV production.
DISCUSSION

Viruses have evolved a number of mechanisms for subverting the host cell translation machinery for their own use. In doing this, they often affect host protein synthesis using mechanisms that include the cleavage of initiation factors or the alteration of their phosphorylation status. For example, during certain picornavirus infections (e.g. with poliovirus or FMDV) eIF4G is cleaved into N-terminal and C-terminal fragments, separating the eIF4E-binding region from the eIF4A and eIF3-binding sites (8–10). Alternatively, infection of cells with another picornavirus, encephalomyocarditis virus, does not induce eIF4G cleavage but leads to dephosphorylation of 4E-BP1, which binds to eIF4E and prevents its interaction with eIF4G (33). Late in adenovirus infection, eIF4E itself is dephosphorylated as a result of the displacement of the eIF4E-kinase Mnk-1 from eIF4G (34). However, viruses have also evolved a number of ways in which to overcome these modifications to the host cell translational machinery. For example, picornaviruses use a cap-independent mechanism of translation initiation that is dependent on an IRES within the 5’-untranslated region of the genome. The viral IRES elements can either bind the ribosome directly or recruit both canonical and noncanonical translation initiation factors to direct translation initiation and can often function with the cleaved forms of initiation factors such as eIF4G (6).

Recent work has highlighted a novel translation initiation mechanism used by members of the Caliciviridae and Potyviridae families (18, 19, 23). This mechanism relies on the interaction of translation initiation factors with a protein (VPg) covalently linked to the 5’ end of the viral genome. These proteins are much larger (13–15 kDa) than the well characterized VPg proteins of picornaviruses (~23 amino acids) and share no sequence homology. It is important to note, however, that whereas the interaction of potyvirus VPg with eIF4E competes with the cap binding activity of eIF4E (22), we have demonstrated that the interaction of calicivirus VPg with eIF4E can occur in the presence of cap (Ref. 19 and current work). This may suggest that although these viruses have evolved similar strategies of VPg-dependent translation initiation, the VPg-eIF4E interaction may occur at different sites on eIF4E.

FIGURE 6. In vitro translation of calicivirus RNA is inhibited by the eIF4A small molecule inhibitor hippuristanol. Rabbit reticulocyte lysates were pretreated with either Me2SO (−) or the eIF4A inhibitor hippuristanol at final concentrations of 1, 0.2, and 0.04 μM. The effect of inhibitor on control bi-cistronic cap-CAT:PTV1 IRES-Luc (A), FCV (B), or MNV RNA (C) was assayed and quantified by phosphorimaging (D). The mean of three experiments is shown with the error bars representing the maximum and minimum values.
During virus infection, several forms of calicivirus VPg are produced; these include the fully processed mature form and precursors where, in the case of FCV, VPg is linked to analogues of the picornavirus 3A and 3CD proteins. It is interesting to note that, as is the case for FCV, where all forms of VPg can be isolated on cap-Sepharose (19), all forms of MNV VPg can also be found in complexes with eIF4E (Fig. 2C). However, the mature and ~32-kDa precursor forms of MNV VPg appear to be enriched in eIF4E-containing complexes (Fig. 2C). The interaction of the potyvirus turnip mosaic virus VPg-containing precursors with eIF4E has also been observed (20). We have previously suggested that one function of the FCV VPg precursor-eIF4E interaction may be to increase the effective concentration of eIF4E within the replication complex (19). It is also possible that other initiation factors are recruited via specific interactions with precursor forms of VPg. It is noteworthy that PABP has been found to interact specifically with VPg-Pro from turnip mosaic virus (20). We have previously failed to observe an interaction between FCV VPg and PABP in vitro (19); however, the interaction of PABP (and other factors) with MNV VPg-containing precursors has yet to be analyzed.

Our observation that MNV VPg interacts with eIF4E is not surprising given our previous observation with LDV VPg, because the two share 51% amino acid identity (data not shown). However, our results on the effect of adding 4E-BP1 or depletion of eIF4E on MNV RNA translation, at least in the rabbit reticulocyte lysate system, brings into question the functional relevance of this interaction for MNV translation. It is possible, but unlikely, that the MNV VPg-eIF4E interaction is merely a remnant of an ancestral initiation mechanism, which is no longer required for efficient translation at least in vitro. A more attractive explanation is that the apparent role of the VPg-eIF4E interaction may be subtle and possibly only observed under more native translation conditions within cells, where the input viral RNA concentration is very limited and has to compete with high concentrations of actively translating host cell mRNA. When a virus particle infects a cell, the viral RNA would be present as a single copy, and under these conditions it is easy to envisage how recruiting eIF4E to the 5' end of the viral RNA may stimulate MNV translation. It is possible that by recruiting eIF4E, other translation initiation factors such as eIF4G, eIF3, and PABP, which may play a more significant role in MNV translation, would also be recruited. The fact that we do not observe any effect in the reticulocyte lysate system may also reflect the relatively high concentrations of initiation factors or the lack of compartmentalization. Indeed, a recent publication has highlighted how the presence of high concentrations of endogenous capped mRNAs, as would be found during the initial stages of infection, can alter the relative contribution of initiation factors and affect the results obtained from in vitro translation reactions (35). Further studies are currently under way to examine the effect that modification or depletion of eIF4E within cells has on calicivirus translation and replication.

The initiation factor eIF4G plays several critical roles in translation initiation but primarily functions as a "scaffold" protein onto which other factors bind, ultimately resulting in the recruitment of the small ribosomal subunit and 43 S preinitiation complex formation (36). Picornaviruses have evolved a mechanism to inhibit host cell cap-dependent translation that is primarily determined by the cleavage of eIF4G by virus-encoded proteases. Cleavage results in the separation of the eIF4E-binding domain from the region that interacts with eIF4A and eIF3 (36), resulting in inhibition of cap-dependent translation. Our results demonstrate that, whereas FCV RNA translation requires intact eIF4E, MNV mRNA translation can occur when eIF4G is cleaved by FMDV Lb protease, suggesting the C-terminal fragment is sufficient or that the eIF4E-eIF4G interaction is not required (Fig. 4). Given that a direct interaction of norovirus VPg with eIF3 has been previously reported (18), one might then predict that eIF3 (and hence the C-terminal fragment of eIF4G, which contains the eIF4A-binding sites) would still be recruited even when eIF4G is cleaved. Unlike our previous observations with FCV, where eIF4G cleavage occurs at late stages of virus replication (37), eIF4G cleavage was not apparent during MNV infection (Fig. 2C). An interaction of MNV VPg with eIF3 has yet to be reported, but as noted above, the relatively high degree of sequence similarity with LDV VPg would make it a high possibility.

eIF4A, the RNA helicase component of the eIF4F complex, is a member of the DEAD box family of putative ATPase/helicases (1). eIF4A is thought to cycle through the eIF4F complex

\(^{7}\) Y. Chaudhry and I. G. Goodfellow, unpublished observations.
Calicivirus Translation Initiation

during initiation (26, 38, 39), where its activity is stimulated by eIF4B and eIF4H (40, 41). eIF4A is recruited to the 5' end of mRNA molecules via an interaction with two binding sites within the middle and C-terminal regions of eIF4G (both in the C-terminal fragment generated by FMDV Lb) and results in an increase in the RNA-stimulated helicase activity of eIF4A (42).

Hippuristatin (25), as well as pateamine (43, 44) both affect eIF4A function, although through different mechanisms. Pateamine affects eIF4A function by deregulating the ATPase and helicase activities, stimulating both (43, 44). Hippuristatin blocks eIF4A-dependent translation by inhibiting its RNA binding, ATPase, and helicase activities via interactions with the C-terminal domain of eIF4A (25). Hippuristatin appears to be highly selective because it has no effect on other RNA and DNA helicases tested and does not affect in vitro RNA splicing reactions (25). The relative requirement of a particular mRNA for eIF4A has been demonstrated to be at least partially determined by the level of RNA structure present in the 5’ end of the mRNA (39). However, it is important to note that the dependence of a particular mRNA on eIF4A is likely to be more complex than this, because previous work has shown that eIF4A can also enhance ribosome binding to unstructured mRNAs (32) including for example AMV-4, a capped viral transcript with little secondary structure (45). Given that our results demonstrate that translation initiation on MNV RNA shows a greater dependence on eIF4A function than FCV RNA, one might predict that the level of secondary structure is significantly greater in MNV RNA than FCV RNA. However, preliminary computational analysis of the RNA structure present at the 5’ end of the calicivirus genomes suggest that this is not the case (data not shown).

Our current study indicates that caliciviruses differ in their functional requirements for translation initiation factors, a different observation made with picornavirus IRES elements. For example, the encephalomyocarditis virus IRES has a high requirement for eIF4A, whereas the PTV-1 IRES shows similarity to the hepatitis C virus IRES in that it does not require any eIF4 initiation factors for function (28). The observation that a small molecule inhibitor of eIF4A inhibits calicivirus replication in cells highlights how this novel paradigm of translation initiation may represent a good target for anti-viral intervention to control outbreaks of this economically important family of viruses.

Acknowledgments—We thank Herbert (Skip) Virgin (Washington University in St. Louis), Tim Skern (University of Vienna), Simon Morley (University of Sussex), Stephen Curry (Imperial College London), Nathum Sonenberg (McGill University), and Craig Cameron (Penn State) for reagents.

REFERENCES

1. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I., and Hellen, C. U. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 7029–7036
2. Gray, N. K., and Wickens, M. (1998) Annu. Rev. Cell Dev. Biol. 14, 399–458
3. Hershey, J. W. B. (1991) Annu. Rev. Biochem. 60, 717–755
4. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–963
5. Kapp, L. D., and Lorsch, J. R. (2004) Annu. Rev. Biochem. 73, 657–704
6. Belsham, G. J., and Jackson, R. J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp 869–900, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Bushell, M., and Sarnow, P. (2002) J. Cell Biol. 158, 395–399
8. Krausslich, H. G., Nicklin, M. J., Toyoda, H., Etchison, D., and Wimmer, E. (1987) J. Virol. 61, 2711–2718
9. Lloyd, R. E., Grubman, M. J., and Ehrenfeld, E. (1988) J. Virol. 62, 4216–4223
10. Devaney, M. A., Vakharina, V. N., Lloyd, R. E., Ehrenfeld, E., and Grubman, M. J. (1988) J. Virol. 62, 4407–4409
11. Lopman, B. A., Reacher, M. H., Van Duijnhoven, Y., Hanon, F. X., Brown, D., and Koopmans, M. (2003) Emerg. Infect. Dis. 9, 90–96
12. Asanaka, M., Atmar, R. L., Ruvelo, V., Crawford, S. E., Neill, F. H., and Estes, M. K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10327–10332
13. Chang, K. O., Kim, Y., Green, K. Y., and Saif, L. J. (2002) Virology 304, 302–310
14. Chang, K. O., Sosnovtsev, S. V., Belliot, G., Kim, Y., Saif, L. J., and Green, K. Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 8733–8738
15. Wobus, C. E., Karst, S. M., Thracklay, L. B., Chang, K. O., Sosnovtsev, S. V., Belliot, G., Krug, A., Mackenzie, J. M., Green, K. Y., and Virgin, H. W. (2004) PLoS Biol. 2, e432
16. Sosnovtsev, S., and Green, K. Y. (1995) Virology 210, 383–390
17. Chang, K.-O., Sosnovtsev, S. S., Belliot, G., Wang, Q., Saif, L. J., and Green, K. Y. (2005) J. Virol. 79, 1409–1416
18. Daughenbaugh, K. F., Fraser, C. S., Hershey, J. J., and Hardy, M. E. (2003) EMBO J. 22, 2852–2859
19. Goodfellow, I., Chaudhry, Y., Gioldosi, I., Gerondopoulos, A., Natoni, A., Labrie, L., Laliberte, J., and Roberts, J. (2005) EMBO Reports 6, 968–972
20. Karst, S. M., Wobus, C. E., Lay, M. D., Davidson, J., and Virgin, H. W. (2003) Science 299, 1575–1578
21. Leonard, S., Plante, D., Wittmann, S., Daigneault, N., Fortin, M. G., and Laliberte, J. F. (2004) J. Gen. Virol. 85, 1053–1063
22. Leonard, S., Chisholm, J., Laliberte, J. F., and Sanfacon, H. (2002) J. Gen. Virol. 83, 2085–2089
23. Leonard, S., Krohn, A., Muller, T., Daigneault, N., Fortin, M. G., and Laliberte, J. F. (2000) J. Virol. 74, 7730–7737
24. Wittmann, S., Chatelat, H., Fortin, M. G., and Laliberte, J. F. (1997) Virology 234, 84–92
25. Karst, S. M., Wobus, C. E., Lay, M. D., Davidson, J., and Virgin, H. W. T. (1999) EMBO J. 18, 4068–4075
26. Karst, S. M., Brierley, I., and Brown, T. D. (1997) J. Gen. Virol. 78, 1033–1040
27. Pestova, T. V., and Kolupaeva, V. G. (2002) Genes Dev. 16, 2906–2922
28. Pestova, T. V., and Kolupaeva, V. G. (2002) Mol. Cell. Biol. 22, 3123–3130
29. Pestova, T. V., Kolupaeva, V. G. (2002) EMBO J. 21, 2394–2404
30. Pestova, T. V., and Kolupaeva, V. G. (2002) EMBO J. 21, 2394–2404
31. Pestova, T. V., and Kolupaeva, V. G. (2002) EMBO J. 21, 2394–2404
32. Pestova, T. V., and Kolupaeva, V. G. (2002) Genes Dev. 16, 2906–2922
33. Pestova, T. V., Kolupaeva, V. G. (2002) Mol. Cell. Biol. 22, 3123–3130
34. Pestova, T. V., Kolupaeva, V. G. (2002) Mol. Cell. Biol. 22, 3123–3130
260, 7651–7658
39. Svitkin, Y. V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G. J., and Sonenberg, N. (2001) RNA 7, 382–394
40. Richter-Cook, N. J., Dever, T. E., Hensold, J. O., and Merrick, W. C. (1998) J. Biol. Chem. 273, 7579–7587
41. Rogers, G. W., Jr., Richter, N. J., and Merrick, W. C. (1999) J. Biol. Chem. 274, 12236–12244
42. Korneeva, N. L., First, E. A., Benoit, C. A., and Rhoads, R. E. (2005) J. Biol.
   Chem. 280, 1872–1881
43. Low, W. K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N. S., Merrick, W. C., Romo, D., and Liu, J. O. (2005) Mol. Cell 20, 709–722
44. Bordeleau, M. E., Matthews, J., Wojnar, J. M., Lindqvist, L., Novac, O., Jankowsky, E., Sonenberg, N., Northcote, P., Teesdale-Spittle, P., and Pelletier, J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10460–10465
45. Gehrke, L., Auron, P. E., Quigley, G. J., Rich, A., and Sonenberg, N. (1983) Biochemistry (Mosc.) 22, 5157–5164