The RNA Helicase eIF4A Is Required for Sapovirus Translation

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The eukaryotic initiation factor 4A (eIF4A) is a DEAD box helicase that unwinds RNA structure in the 5’ untranslated region (UTR) of mRNAs. Here, we investigated the role of eIF4A in porcine sapovirus VPg-dependent translation. Using inhibitors and dominant-negative mutants, we found that eIF4A is required for viral translation and infectivity, suggesting that despite the presence of a very short 5’ UTR, eIF4A is required to unwind RNA structure in the sapovirus genome to facilitate virus translation.

Eukaryotic initiation factor 4F (eIF4F), comprised of eIF4E, eIF4A, and eIF4G, is essential for initiation of cellular protein synthesis (1). eIF4E binds the cap structure on the 5’ end of mRNAs, while eIF4A, an RNA helicase, unwinds secondary structure in the 5’ untranslated region (UTR), facilitating ribosomal recruitment (1). eIF4G coordinates the assembly of the eIF4F complex and the recruitment of additional eIFs (1, 2).

The expression of viral proteins is frequently regulated at the level of translation initiation (2). Members of the Caliciviridae family of positive-sense RNA viruses use a novel mechanism of viral protein synthesis that relies on the interaction of initiation factors with a virus-encoded protein, VPg, covalently linked to the 5’ end of viral RNAs (3–5). The VPg proteins of feline calcivirus (FCV), murine norovirus (MNV), and porcine sapovirus (PSaV) interact with the eIF4F complex in infected cells (3, 4, 6). However, the functional roles of the components of the eIF4F complex differ among caliciviruses (3); VPg from all three viruses binds eIF4A, and eIF4G, is essential for initiation of cellular protein synthesis (1), and eIF4E is required to unwind RNA structure in the sapovirus genome to facilitate virus translation.

To understand the roles of eIF4F components in sapovirus translation, we investigated the role of eIF4A in the PSaV life cycle. PSaV is a member of the Sapovirus genus of the Caliciviridae family and remains the only member of the genus capable of replication in cell culture (7, 8). PSaV is used as a model to study the mechanisms of sapovirus genome translation and replication.

eIF4A is required for the translation of FCV and MNV (3), even though they have very short 5’ UTRs (4 to 19 bases). Using the secondary structure prediction algorithm Mfold (9), a high degree of RNA secondary structure at the 5’ end of the PSaV genome was predicted (Fig. 1A). The open reading frame 1 (ORF1) start codon is predicted to be located in the first stem, with a calculated stability of \( \Delta G = -10.8 \text{kcal/mol} \). The presence of five stable stem-loops (SLs) in the 5’ end was experimentally confirmed using selective 2’-hydroxyl acylation and primer extension (SHAPE) analysis. In vitro-transcribed PSaV RNA was modified by incubation with N-methyl isatoic anhydride (NMIA). Highly modified bases, characteristic of unstructured RNA, were detected by stalling in a reverse transcriptase reaction using \( { }^{32}\text{P}-\text{labeled primers. Labeled cDNA products were separated on a denaturing 6% acrylamide gel and detected on a phosphorimager (Fig. 1B). Again, using Mfold, the start codons of other sapoviruses are shown to be similarly trapped in stems ranging in stability from \( \Delta G = -8.7 \) to \( \Delta G = -19.2 \text{kcal/mol} \) (Fig. 1C). The ATPase and helicase activities of eIF4A are sufficient for ribosomal scanning of 5’ UTRs with a weak to moderate secondary structure (\( \Delta G = -13.1 \text{kcal/mol} \) or weaker) (10). The structure present in the region spanning the short 5’ UTR and the viral polyprotein coding sequences in the PSaV genome suggests that eIF4A may play a role in modifying the structure of the viral RNA to efficiently initiate virus translation (11, 12).

To characterize the role of eIF4A in the PSaV translation, the effect of hippuristanol, a specific eIF4A inhibitor (13, 14), on PSaV translation in vitro was examined in rabbit reticulocyte lysates (RRLs) programmed with viral VPg-linked RNA obtained from PSaV-infected cells (6). Hippuristanol is a polyoxynated steroid that specifically inhibits the RNA binding, RNA-dependent ATPase, and helicase activities of eIF4A (13). RNA from PSaV-infected cells and in vitro-transcribed RNA from a dicistronic construct expressing cap-dependent chloramphenicol acetyltransferase (CAT) and porcine teschovirus (PTV)-internal ribosome entry site (IRES)-dependent luciferase were subjected to an in vitro translation reaction. Hippuristanol inhibited PSaV translation in a dose-dependent manner. As expected, cap-dependent CAT expression was also inhibited, but the PTV-IRES-dependent luciferase translation, which is eIF4A independent, was unaffected (Fig. 2A).

We also examined the effect of wild-type (wt) or three dominant-negative mutants of eIF4AI, E183Q (DQAD), R362Q (PRRVA12), and R362Q (15–17), on
PsVaV translation in vitro. While the addition of increasing concentrations of wt eIF4A slightly enhanced PsVaV translation, all dominant-negative mutants inhibited PsVaV translation (Fig. 2B to E). As expected, all eIF4A mutants reduced cap-dependent CAT translation, whereas eIF4F-independent PTV-IRES translation was slightly increased, possibly as a result of increased availability of other eIFs being redirected away from cap-dependent initiation (Fig. 2B to E).

The possibility of a direct interaction between the PsVaV VPg protein and eIF4A was examined using His tag pulldown assays; however, no interaction was observed (data not shown). To investigate if eIF4A was associated with viral RNA during replication, coimmunoprecipitation of viral RNA with eIF4A was performed. Antibodies to eIF4A and VPg, as a control, were able to coimmunoprecipitate significantly more PsVaV viral RNA than the control antibody (Fig. 3A). While eIF4A immunoprecipitated from infected cells could be either free or eIF4F-associated, we next examined if eIF4A could bind to the

![Diagram of secondary structure](image-url)
PSaV RNA directly. To examine this and confirm the specificity of interaction, recombinant His-tagged eIF4AI or the C-terminal fragment of eIF4GI (amino acids [aa] 1118 to 1600) was used to precipitate PSaV or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA, from RNA preparations isolated from infected cells, in the presence of the nonhydrolyzable ATP analogue adenylylimidodiphosphate (AMP-PNP), a hallmark of eIF4AI binding. eIF4AI precipitated PSaV RNA significantly, however, neither proteins precipitated GAPDH RNA (Fig. 3B), which would be expected to bind only eIF4F-associated eIF4AI. The interaction between recombinant eIF4A and PSaV RNA was also found to be sensitive to hippuristanol (Fig. 3C), consistent with the findings in Fig. 2 and Fig. 4A, confirming a direct specific interaction between eIF4A and the PSaV genome.

To examine the functional role for eIF4A in the PSaV life cycle,
the effect of hippuristanol on PSaV replication in cell culture was examined. The PSaV titer was reduced by hippuristanol in a dose-dependent manner (Fig. 4A) at concentrations where cell viability was unaffected (data not shown). This suggests that PSaV VPg-dependent translation may be more susceptible to eIF4A inhibition than canonical cellular translation, although potential contributions from pleotropic effects of the drug cannot be excluded. Depletion of eIF4AI by RNA interference (Fig. 4B) resulted in significantly reduced PSaV genome levels and yields of infectious virus (Fig. 4C).
virus (Fig. 4C and D). Together with our in vitro analysis, our results demonstrate a functional role for eIF4A in the PSaV life cycle, providing additional insight into the novel mechanism of protein-primed translation initiation and the life cycle of poorly characterized caliciviruses.

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REFERENCES

1. Hinnebusch AG. 2014. The scanning mechanism of eukaryotic translation initiation. Annu Rev Biochem 83:779–812. http://dx.doi.org/10.1146/annurev-biochem-060713-035802.

2. Lopez-Lastra M, Ramdohr P, Letelier A, Vallejos M, Vera-Otarola J, Valiente-Echeverria F. 2010. Translation initiation of viral mRNAs. Rev Med Virol 20:177–195. http://dx.doi.org/10.1002/rmv.649.

3. Chaudhry Y, Nayak A, Bordelaeau M-E, Tanaka J, Pelletier J, Belsham GJ, Roberts LO, Goodfellow IG. 2006. Caliciviruses differ in their functional requirements for eIF4F components. J Biol Chem 281:25315–25325. http://dx.doi.org/10.1074/jbc.M602230200.

4. Goodfellow I, Chaudhry Y, Gioldasi I, Gerondopoulos A, Natoni A, Labrie L, Laliberte JF, Roberts L. 2005. Calicivirus translation initiation requires an interaction between VPg and eIF4E. EMBO Rep 6:966–972. http://dx.doi.org/10.1038/sj.embor.7400510.

5. Daughenbaugh KF, Wobus CE, Hardy ME. 2006. VPg of murine norovirus binds translation initiation factors in infected cells. Virol J 3:33–39. http://dx.doi.org/10.1186/1743-422X-3-33.

6. Hosmillo M, Chaudhry Y, Kim DS, Goodfellow I, Cho KO. 2014. Sapovirus translation requires an interaction between VPg and the cap binding protein eIF4E. J Virol 88:12213–12221. http://dx.doi.org/10.1128/JVI.01650-14.

7. Chang KO, Sosnovtsev SV, Belliot G, Kim Y, Saif LJ, Green KY. 2004. Bile acids are essential for porcine enteric calicivirus replication in association with down-regulation of signal transducer and activator of transcription 1. Proc Natl Acad Sci USA 101:8733–8738. http://dx.doi.org/10.1073/pnas.0401126101.

8. Chang KO, Sosnovtsev SS, Belliot G, Wang QH, Saif LJ, Green KY. 2005. Reverse genetics system for porcine enteric calicivirus, a prototype sapovirus in the Caliciviridae. J Virol 79:1409–1416. http://dx.doi.org/10.1128/JVI.79.3.1409-1416.2005.

9. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415. http://dx.doi.org/10.1093/nar/gkg595.

10. Pisareva VP, Pisarev AV, Komar AA, Hellen CU, Pestova TV. 2008. Translation initiation on mammalian mRNAs with structured 5′UTRs requires DExH-box protein DHX29. Cell 135:1237–1250. http://dx.doi.org/10.1016/j.cell.2008.10.037.

11. Yu Y, Abaeva IS, Marintchev A, Pestova TV, Hellen CU. 2011. Common conformational changes induced in type 2 picornavirus IRESes by cognate trans-acting factors. Nucleic Acids Res 39:4851–4865. http://dx.doi.org/10.1093/nar/gkr045.

12. Chu J, Pelletier J. 2015. Targeting the eIF4A RNA helicase as an anti-neoplastic approach. Biochim Biophys Acta 1849:781–791. http://dx.doi.org/10.1016/j.bbabmbre.2014.09.006.

13. Bordelaeau ME, Mori A, Oberer M, Lindqvist I, Chard LS, Higa T, Belsham GJ, Wagner G, Tanaka J, Pelletier J. 2006. Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. Nat Chem Biol 2:213–220. http://dx.doi.org/10.1038/nchembio776.

14. Lindqvist I, Oberer M, Reibarkh M, Cencic R, Bordelaeau M-E, Vogt E, Marintchev A, Tanaka J, Fagotto F, Altmann M, Wagner G, Pelletier J. 2008. Selective pharmacological targeting of a DEAD box RNA helicase. PLoS One 3:e1583. http://dx.doi.org/10.1371/journal.pone.0001583.

15. Pause A, Methot N, Svitkin Y, Merrick WC, Sonenberg N. 1994. Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. EMBO J 13:1205–1215.

16. Pause A, Methot N, Sonenberg N. 1994. The HRIGRXXXR region of the DEAD box RNA helicase eukaryotic translation initiation factor 4A is required for RNA binding and ATP hydrolysis. Mol Cell Biol 15:6789–6798. http://dx.doi.org/10.1128/MCB.13.11.6789.

17. Svitkin YV, Pause A, Haghighat A, Pyronnet S, Witherell G, Belsham GJ, Sonenberg N. 2001. The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5′ secondary structure. RNA 7:382–394. http://dx.doi.org/10.1017/S135583820010108X.