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Coxsackievirus B3 2A protease promotes encephalomyocarditis virus replication

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ABSTRACT

To determine whether 2A protease of the enterovirus genus with type I internal ribosome entry site (IRES) effect on the viral replication of type II IRES, coxsackievirus B3(CVB3)-encoded protease 2A and encephalomyocarditis virus (EMCV) IRES (Type II)-dependent or cap-dependent report gene were transiently co-expressed in eukaryotic cells. We found that CVB3 2A protease not only inhibited translation of cap-dependent reporter genes through the cleavage of eIF4GI, but also conferred high EMCV IRES-dependent translation ability and promoted EMCV replication. Moreover, deletions of short motif (aa13–18 RVVNRH, aa65–70 KNKHYP, or aa88–93 PRRYQSH) resembling the nuclear localization signals (NLS) or COOH-terminal acidic amino acid motif (aa133–147 DIRDLLWLEDDAMEQ) of CVB3 2A protease decreased both its EMCV IRES-dependent translation efficiency and destroy its cleavage on eukaryotic initiation factor 4G (eIF4G) I. Our results may provide better understanding into more effective interventions and treatments for co-infection of viral diseases.

1. Introduction

Cap-dependent and internal ribosome entry site (IRES) elements are two major mechanisms involved in the initiation of mRNA translation in eukaryotic cells. In eukaryotes, protein synthesis requires a modified nucleotide 'cap' on the mRNA and proteins that recruit and position the ribosome (Merrick, 2004; Pestova et al., 2001; Pisarev et al., 2005; Sonenberg and Hinnebusch, 2009).

Many pathogenic viruses also use an alternative, cap-independent mechanism (IRES) that substitutes RNA structure to translate proteins. Some IRESs are able to bind directly to the ribosome through a specific RNA structure (Doudna and Sarnow, 2007; Hellen and Sarnow, 2001; Jackson, 2005). In animals, major groups of viruses encoding mRNAs with IRES elements include picornaviruses, dicistrovirus, flaviviruses, pestiviruses, and retroviruses (Balvay et al., 2009; Bienz et al., 1982; Jang, 2006; Nakashima and Uchiumi, 2009). These viral IRESs have been classified into five major structural groups (Asnani et al., 2015). IRES type I is typical of entero/rhinoviruses with poliovirus as the prototype, while type II is present in cardio/aphthoviruses, the prototype being encephalomyocarditis virus (EMCV). Hepatitis A virus is Type III, and Hepatitis C virus and Classical swine fever virus (CSFV) is Type IV (Asnani et al., 2015), Type V IRESs were recently identified in members of the genera Kobuvirus, Salivirus, and Oscivirus (Sweeney et al., 2012; Yu et al., 2011). Up to five groups may be present in picornaviruses, although there are two that are more representative (Asnani et al., 2015; Belsham, 2009; Hellen and Wimmer, 1995).

Footnote: internal ribosome entry site (IRES), encephalomyocarditis virus (EMCV), untranslated regions (UTR), cricket paralysis virus (CrPV), eukaryotic initiation factor 4G (eIF4G), classical swine fever virus (CSFV).

The Picornaviridae family includes a large number of animal pathogens, which are the causative agents of important diseases found worldwide. Their genomes consist of similarly organized single sense-strand RNA molecules, where untranslated regions (UTR) flank a single open reading frame that encodes a large polyprotein. The 5′ end of the viral RNA is covalently linked to a small viral polypeptide, VPg, while the 3′ end is polyadenylated (Martinez-Salas et al., 2008). However, each picornavirus genera is defined by its unique features found in the UTR and coding regions (Oberste

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et al., 2007). Genomes of picornaviruses as well as other positive strand RNA viruses possess distinct functions in the viral life cycle. These include viral protein synthesis, negative strand RNA replication, and viral packaging into virions. To perform these multiple roles, regulatory mechanisms have evolved to finish these functions during the different stages of the viral cycle. Many of these regulatory mechanisms rely on structural elements located in the non-coding regions of the genome, in which specialized motifs are recognized by RNA-binding proteins encoded by the host or the viral RNA. The genome of each genus also contains distinctive features found along the coding region. One of the differences among members of the Picornaviridae family involves the virus-encoded proteases.

The encoded 2A protease by enteroviruses differs structurally and functionally from other 2A proteins by members of other genera. As an example, the 2A protein derived from cardioviruses lack sequence homology to the 2A protease of enterovirus and protease activity. The 2A protease of enterovirus and rhinovirus are cysteine proteinases which share significant sequence homology to the trypsin-like small serine proteases. Enterovirus 2A protease processes the viral polyprotein and various other host proteins. These include eukaryotic initiation factor 4G (eIF4G) I, eIF4GII, and poly(A)-binding protein. It has been shown that during enterovirus infection, the 2A protease inhibits host translation and modulates viral RNA replication, viral translation, and viral RNA stability (Morrison and Racaniello, 2009).

There are unique structural and mechanistic classes of IRES, which remains undetermined to whether 2A protease facilitates other type IRES dependent translation and promotes other genera virus replication. Here, we report that the 2A protease of Coxsackievirus B3 (CVB3), which is an enterovirus of the picornavirus family with IRES type I, confers high efficiency EMCV IRES (Type II)-dependent protein translation, inhibits classical cap-dependent protein translation, and promote EMCV replication. Similar to poliovirus 2A protease, expression of CVB3 2A protease led to cleavage of eIF4G I, a key factor for host protein synthesis. Moreover, deletions of short motif (aa13–18 RVVNRH, aa65–70 KNKHYP, or aa88–93 PRRYQSH) resembling the nuclear localization signals (NLS) or COOH-terminal basic amino acid motif (aa133–147 DIRDLLWLEDDAMEQ) of CVB3 2A protease decrease its activity to inhibit EMCV IRES-dependent translation. We have also observed that 2A protease localized in perinuclear membrane and cytoplasm, and deletions of any NSL-like sequences prevented its localization to perinucleus. Deletion of C-terminal aa133–147 of the protease 2A facilitated its nuclear localization. These results demonstrated CVB3 2A protease upregulated EMCV IRES-dependent viral genome translation over cap-dependent host mRNA translation and facilitated EMCV replication by cross genera. A previous study has shown that there were apparent coinfections with more than one picornavirus in a single patient stool specimens from acute flaccid paralysis (AFP) cases and healthy contacts (Nix et al., 2013). Our results may provide better understanding into more effective interventions and treatments for co-infection of two picornavirus.

2. Materials and methods

2.1. Cell lines and virus

HeLa, 293T, and BHK-21 cells were cultured in double modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. CVB3 (Nancy strain) was propagated and titrated on HeLa cell monolayer. Cells were seeded on culture dishes a day before infection with CVB3 at 1× TCID50. Cells were then incubated at 37 °C throughout the infection. To determine the effects of CVB3 2A protease on the replication of EMCV(BJC3), 5×10^4 BHK-21 cells were used to inoculate with 1×, or 10× TCID50 EMCV in 96-well plates. After 1 h adsorption, the cell layer was rinsed with 1× PBS and DMEM containing 2% FBS was added. For virus RNA copy numbers determination, BHK-21 cells per well were grown for 12 h in DMEM with 2% FBS.

2.2. Real-time reverse transcriptase-polymerase chain reaction

To quantify EMCV RNA copy numbers, cells inoculated with virus were collected, and viral RNAs were isolated using a QIAamp viral RNA minikit (Qiagen, Germany). Real-time quantitative RT-PCR (RT-qPCR) was performed using the one-step reaction mixture (ABI) containing 100 ng of each primer (forward, 5′-GGTGAGAGCAAGCCTCGCAAAGACAG-3′ and reverse, 5′-CCCTACCTCACGGAATGGGGCAAAG-3′) and 0.8 μl of probe (5′-FAM-TCGGCCTGTCATGAACAGAGAGGCG-Tamara-3′). Reaction conditions were 48 °C for 30 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All further analysis was done using Biorad Software (Biorad CFX96, USA).

2.3. Plasmid construction

The cDNAs encoding CVB3 2A protease were obtained by reverse transcription PCR of total RNA from CVB3-infected HeLa cells. The DNA was cloned into *EcoR*I and *Hind*III restriction sites of the eukaryotic expression vector pcDNA3.1 formed vector p2A which produce Flag-tagged proteins. The three DNA fragments encoding CVB3 2A protease with the basic amino acid-rich region truncation of 13–18 (RVVNRH), 65–70 (KNKHYP), and 88–93 (PRRYQS) amino acids and the one fragment with acidic amino acid-rich truncation of 133–147 (DIRDLLWLEDDAMEQ) were also cloned, respectively, into pcDNA3.1 to form plasmids p2AΔ13, p2AΔ65, p2AΔ88, and p2AΔ133 with Flag-tag (Fig. 1). Plasmid pFluc–EMCV–IRES–Rluc was constructed by inserting the EMCV IRES and Fluc fragment into plasmid pRluc. Plasmid pEMCV–IRES–EGFP was inserted with EMCV IRES sequences following with GFP sequences. Plasmid pEGFP-N1 with CMV promoter and GFP sequences was purchased from Promega Corporation (USA). All of the expression plasmids were verified by DNA sequencing.

2.4. Immunofluorescence assay staining

293T and BHK-21 cells were seeded at a density of about 2.5×10^5 cells per 35 mm culture dish. After overnight incubation, cells were transfected with plasmids (0.5–2 μg) using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, USA). After 48 h of transfection, GFP expressions in cells was assessed by Fluorescence microscopy (Nikon, Japan). Transfected cells were fixed with acetone for 10 min, washed with buffer (1% skim milk in PBS) twice for 5 min each, and then incubated with the anti-Flag antibody (1:200 dilution) at 37 °C for 30 min. Cells were washed with PBS at room temperature for 5 min three times, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:2000 dilution) at 37 °C for 30 min. Cells were washed three more times with PBS, and then DAPI (Merck, Germany) was used to stain the nucleus.

2.5. Luciferase assay

Approximately 4×10^5 BHK-21 cells were plated on a 96-well tissue culture Plates 3 h before transfection. Cells were cotransfected with pFluc–EMCV–IRES–RLuc (0.05 μg/well) and p2A/pcDNA3.1 (0.2 μg/well). Luciferase activity was measured by the dual-luciferase assay system (Promega, USA) according to the