Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Assessing activity of Hepatitis A virus 3C protease using a cyclized luciferase-based biosensor

Junwei Zhou [a], Dang Wang [a, b, *], Yongqiang Xi [a, b], Xinyu Zhu [a, b], Yuting Yang [a, b], Mengting Lv [a, b], Chuanzhen Luo [a], Jiyao Chen [a, b], Xu Ye [a, b], Liurong Fang [a, b], Shaobo Xiao [a, b]

[a] State Key Laboratory of Agricultural Microbiology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
[b] The Cooperative Innovation Center for Sustainable Pig Production, Wuhan 430070, China

ARTICLE INFO

Article history:
Received 26 April 2017
Accepted 10 May 2017
Available online xxx

Keywords:
Firefly luciferase
Biosensor
Hepatitis A virus
3C protease
Intein

ABSTRACT

Hepatitis A is an acute infection caused by Hepatitis A virus (HAV), which is widely distributed throughout the world. The HAV 3C cysteine protease ($3C^{pro}$), an important nonstructural protein, is responsible for most cleavage within the viral polyprotein and is critical for the processes of viral replication. Our group has previously demonstrated that HAV $3C^{pro}$ cleaves human NF-κB essential modulator (NEMO), a kinase required in interferon signaling. Based on this finding, we generated four luciferase-based biosensors containing the NEMO sequence (PVLKAQ↓ADIYKA) that is cleaved by HAV $3C^{pro}$ and/or the *Nostoc punctiforme* DnaE intein, to monitor the activity of HAV $3C^{pro}$ in human embryonic kidney cells (HEK-293T). Western blotting showed that HAV $3C^{pro}$ recognized and cleaved the NEMO cleavage sequence incorporated in the four biosensors, whereas only one cyclized luciferase-based biosensor (233-DnaE-HAV, 233DH) showed a measurable and reliable increase in firefly luciferase activity, with very low background, in the presence of HAV $3C^{pro}$. With this biosensor (233DH), we monitored HAV $3C^{pro}$ activity in HEK-293T cells, and tested it against a catalytically deficient mutant HAV $3C^{pro}$ and other virus-encoded proteases. The results showed that the activity of this luciferase biosensor is specifically dependent on HAV $3C^{pro}$. Collectively, our data demonstrate that the luciferase biosensor developed here might provide a rapid, sensitive, and efficient evaluation of HAV $3C^{pro}$ activity, and should extend our better understanding of the biological relevance of HAV $3C^{pro}$.

1. Introduction

Hepatitis A virus (HAV) is the commonest cause of clinically apparent viral hepatitis. Almost 1.4 million new cases of HAV infection are estimated to occur globally each year, with 11%–22% requiring hospitalization [1–3]. HAV is a small nonenveloped RNA virus with a single-stranded RNA genome of approximately 7500 nucleotides, and is a member of the family Picornaviridae. The single large open reading frame in the HAV genome is divided into three functional regions, designated P1, P2, and P3. The P1 region encodes the capsid polypeptides (VP1, VP2, and VP3), and the P2 and P3 regions encode the nonstructural polypeptides, which are predicted to be processed into mature viral proteins by viral proteases [1,4–7]. The HAV 3C cysteine protease ($3C^{pro}$) is responsible for most cleavage within the viral polyprotein, supporting viral replication and proliferation [1,7,8].

Its pivotal role in viral replication and proliferation makes HAV $3C^{pro}$ one of the major targets in the design of *anti*-HAV drugs [6]. The traditional screening methods used to detect HAV $3C^{pro}$ activity are so slow and inefficient that they do not meet the requirements for high-throughput screening. Therefore, a simple, efficient, high-throughput method for the detection of HAV $3C^{pro}$ activity at the cell level, which fully reflects the characteristic biological activity of the protease, is urgently required. Compared with fluorescence-based assays, bioluminescence-based assays are markedly more sensitivity, with a wider dynamic range in detecting the activities of proteases [9–11]. Firefly luciferase is a 61-kDa monomeric enzyme that catalyzes the oxidation of firefly luciferin, which then emits a yellow-green light in the presence of ATP and oxygen [12,13]. As a

* Corresponding author. Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, 1 Shi-zi-shan Street, Wuhan 430070, Hubei, China.
E-mail address: wangdang511@126.com (D. Wang).

http://dx.doi.org/10.1016/j.bbrc.2017.05.063
0006-291X/

reporter protein, firefly luciferase is widely used to detect apoptosis and enzyme activity, and to screen for antiapoptotic drugs and identify enzyme recognition sequences [14–16]. Previous research has shown that DNA polymerase III intein (DnaE) from *Nostoc punctiforme* (Npu) catalyzes the protein *trans*-splicing reaction at an extraordinarily high rate and is widely used in the cyclization of proteins without affecting their activities [17–19]. Therefore, DnaE was fused to a randomly mutated thermally stable firefly luciferase construct, modified at residue 358 or 233, to increase the sensitivity of the firefly luciferase assay of apoptosis. In a series of experiments, these recombined luciferase reporters displayed increased sensitivity and yielded reproducible data with low background [16,20].

Recently, our group has previously shown that HAV $3C^{pro}$ cleaves the glutamine residue at position 304 (Gln304) of human NF-κB essential modulator (NEMO) to inhibit the expression of type I interferon (IFN) [21]. Therefore, in this study, we combined the HAV $3C^{pro}$ cleavage recognition sequence of NEMO with a simple reporter system involving firefly luciferase to produce a cell-based protease reporter system, generating the reporters 233-HAV (233H), 358-HAV (358H), 233-DnaE-HAV (233DH), and 358-DnaE-HAV (358DH). The cyclized firefly luciferase protein expressed by 233DH was successfully cleaved by HAV $3C^{pro}$, resulting in a significant increase in luciferase activity. The luciferase activity of 233DH was confirmed to be specifically dependent on HAV $3C^{pro}$ activity, but not on the protease activities of other viruses, including the noroviruses (NoV), severe acute respiratory syndrome coronavirus (SARS coronavirus, SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), Human coronavirus 229E (HCoV-229E), porcine epidemic diarrhea virus (PEDV), and porcine deltacoronavirus (PDCoV), confirming the specificity of 233DH for HAV $3C^{pro}$ activity. Taken together, we might have developed a rapid and sensitive method to detect HAV $3C^{pro}$ activity in HEK-293T cells.

2. Materials and methods

2.1. Cells

Human embryonic kidney cells (HEK-293T) and human hepatoma cells (Huh-7), obtained from the China Center for Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under 5% CO_2 in a humidified incubator.

2.2. Plasmids

The cDNA expression constructs encoding wild type HAV $3C^{pro}$ and its catalytically inactive mutant (C172A) have been described previously [21]. The 3C-like protease ($3CL^{pro}$) genes from NoV, SARS-CoV, MERS-CoV, HCoV-229E, PEDV, and PDCoV were also synthesized/amplified and cloned into pCAGGS-HA-C.

The DNA sequence encoding the conserved residues in DnaE [20] was synthesized and cloned into the pCAGGS-MCS vector to construct pCAGGS-DnaE. The sequences encoding the C-terminal fragment (amino acids 235–544) and the N-terminal fragment (amino acids 4–233) of firefly luciferase were amplified from the firefly luciferase reporter vector pGL4.21luc2P/Puro (Promega, USA), respectively. With two-step overlapping PCR, the sequences encoding amino acids 4–233 and 235–544 were cloned together into the pCAGGS-MCS vector, to generate the pCAGGS-233 vector. The sequences encoding other N-terminal (amino acids 4–354) and C-terminal fragments (amino acids 358–544) of firefly luciferase were also amplified and cloned to generate the pCAGGS-358 vector. Oligonucleotides corresponding to the amino acid sequence PVLKAQ↓ADIYKA of NEMO (cleaved by HAV $3C^{pro}$) or ENLYFQ↓S (cleaved by Tobacco etch virus (TEV) $3C^{pro}$) [13,21] was also ligated into the pCAGGS-233 vector to construct the 233H reporter or the control 233 reporter, respectively. The sequence encoding amino acids 4–233 and 235–544 fused to the corresponding amino sequence PVLKAQ↓ADIYKA or ENLYFQ↓S was cloned into pCAGGS-DnaE, to generate the 233DH reporter or the control 233D reporter, respectively. The same construction strategy was used to generate the 358H reporter, the 358DH reporter, and their control reporters 358 and 358D, respectively. The sequences of biosensor expression plasmids are shown in Table S1.

2.3. Western blotting analysis

Briefly, HEK-293T cells cultured in 60 mm dishes were transfected with the various plasmids. After 30 h, the cells were harvested by the addition of lysis buffer, and the protein concentrations were measured in the whole-cell extracts. The proteins in the extracts were separated with 12% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with antibodies and secondary antibodies. To confirm the expression levels of HA-tagged wild-type (WT) and mutant $3C^{pro}$, an anti-HA antibody (MBL, Japan) was used in an immunoblotting analysis. An anti-goat monoclonal secondary antibody (Promega, USA) was used to detect the expression of each luciferase reporter. The expression of β-actin was monitored with an *anti*-β-actin mouse monoclonal antibody (Beyotime, China) to confirm equal protein loading.

2.4. Luciferase reporter gene assays

The luciferase reporter constructs 233H, 358H, 233DH, 358DH and their controls (233, 358, 233D, 358D respectively) were used to detect HAV $3C^{pro}$ activity. HEK-293T cells or Huh-7 cells plated in 48-well plates were transfected with various $3C^{pro}$ and $3CL^{pro}$ expression plasmids or the empty control plasmid, together with the luciferase reporter plasmid and pRL-TK (Promega) (used as an internal control to normalize transfection efficiency). The cells were lysed 36 h later, and the firefly luciferase and Renilla luciferase activities were determined with a luciferase reporter assay system (Promega, USA), according to the manufacturer's protocol. The data represent the relative firefly luciferase activities normalized to the corresponding Renilla luciferase activities.

2.5. Statistical analysis

The results are presented as the means ± standard deviations (SD) of at least three experiments. Significant differences were detected with Student's *t*-test. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; ns, not significant).

3. Results

3.1. Generation of four biosensors to evaluate HAV $3C^{pro}$ activity

As described in our previous study, HAV $3C^{pro}$ recognizes and cleaves human NEMO at the Gln304 residue [21]. Thus, we combined the HAV $3C^{pro}$ cleavage recognition sequence (PVLKAQ↓ADIYKA) of NEMO with a firefly luciferase reporter system to monitor the activity of HAV $3C^{pro}$ in HEK-293T cells. To this end, pCAGGS-233 and pCAGGS-358 were fused to the cleavage sequence of NEMO recognized by HAV $3C^{pro}$ to generate 233H and 358H, respectively (Fig. 1A). As described previously, DnaE is widely used in the cyclization of proteins because this cyclization does not

Please cite this article in press as: J. Zhou, et al., Assessing activity of Hepatitis A virus 3C protease using a cyclized luciferase-based biosensor, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.05.063