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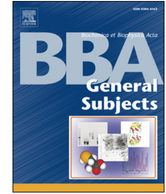

Glutathionylation of chikungunya nsP2 protein affects protease activity

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ABSTRACT

Background: Chikungunya fever is an emerging disease caused by the chikungunya virus and is now being spread worldwide by the mosquito *Aedes albopictus*. The infection can cause a persistent severe joint pain and recent reports link high levels of viremia to neuropathologies and fatalities. The viral protein nsP2 is a multifunctional enzyme that plays several critical roles in virus replication. Virus infection induces oxidative stress in host cells which the virus utilizes to aid viral propagation. Cellular oxidative stress also triggers glutathionylation which is a post-translational protein modification that can modulate physiological roles of affected proteins.
Methods: The nsP2 protease is necessary for processing of the virus nonstructural polyprotein generated during replication. We use the recombinant nsP2 protein to measure protease activity before and after glutathionylation. Mass spectrometry allowed the identification of the glutathione-modified cysteines. Using immunoblots, we show that the glutathionylation of nsP2 occurs in virus-infected cells.
Results: We show that in virus-infected cells, the chikungunya nsP2 can be glutathionylated and we show this modification can impact on the protease activity. We also identify 6 cysteine residues that are glutathionylated of the 20 cysteines in the protein.
Conclusions: The virus-induced oxidative stress causes modification of viral proteins which appears to modulate virus protein function.
General significance: Viruses generate oxidative stress to regulate and hijack host cell systems and this environment also appears to modulate virus protein function. This may be a general target for intervention in viral pathogenesis.

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1. Introduction

Chikungunya virus (CHIKV) is an alphavirus of the family *Togaviridae*, a classification based on the genomic organization, amino acid sequences and replication strategies [1]. CHIKV is transmitted by mosquitoes, especially *Aedes aegypti* and *Aedes albopictus* and is the causative agent of chikungunya fever. CHIKV infection causes an acute febrile illness, most often characterized by high fever, headache, fatigue, nausea, vomiting, myalgia, rash and severe arthralgia. The arthralgia, or joint pain, can be severe and extremely painful while persisting for months or even years with the morbidity preventing a return to employment [2,3]. Within the last decade, large outbreaks have occurred worldwide with >2 million cases in Africa and around the Indian Ocean [4,5] as well as more recently in the Americas with more than a million suspected cases of chikungunya fever having been reported [6]. Recent reports have suggested that high levels of CHIKV viremia are linked to neuropathologies [7–9], and mouse models corroborate this with severe infection involving the central nervous system [10,11]. Chikungunya infection therefore, can lead to increased neuropathies as well as severe systemic syndromes and fatality [7,12].

The approximately 12 kb CHIKV genome consists of 2 open reading frames for translation of nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) and structural proteins (C, E1, E2, E3 and 6 K) [13]. Individual nonstructural proteins, nsP1, nsP2, nsP3 and nsP4, possess unique functions that are important for viral replication and propagation [14]. The processing of the long nonstructural polyprotein into separate proteins is performed by the protease activity of the viral nsP2 protein [15]. Additionally, the viral nsP2 protein has other enzymatic functions including RNA helicase, nucleoside triphosphatase (NTPase) and RNA-dependent 5′-triphosphatase activities which are located at the N-terminus of the protein, while the protease domain is located at the C-terminus [16,17]. Previous studies have shown that the nsP2 proteolytic processing of the nonstructural polyprotein is highly regulated with cleavage occurring in a specific order [18]. In addition to the multiple enzyme activities directly involved in viral replication, nsP2 also has

Abbreviations: CHIKV, Chikungunya virus; SINV, Sindbis virus; SFV, Semliki Forest virus; VEEV, Venezuelan equine encephalitis virus; nsP2, Nonstructural protein 2; MBP, Maltose-binding protein; AGA, Cleavage sites nsP1/nsP2; AGC, nsP2/nsP3; AGG, nsP3/nsP4; (hpi), Hours post-transfection.

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been shown to translocate to the nucleus for unknown functions, as well as interact with multiple host cell proteins to enhance virus production [19–26]. With these multiple diverse roles nsP2 would appear to be a critical component for alphavirus replication.

Many viruses have been shown to alter cellular homeostasis and induce oxidative stress early in infection [27,28] as has CHIKV [29,30]. Within the cell, oxidative stress can lead to an important oxidative modification called glutathionylation whereby specific redox-sensitive cysteines in proteins are modified by the addition of endogenous glutathione (GSH) in response to the stress [31–33]. Glutathionylation has been shown to exert structural and functional effects on targeted proteins with various consequences such as inhibition or activation of biological activity [34]. In cells, deglutathionylation of proteins is mainly enzymatically catalyzed by glutaredoxin although thioredoxin and sulfiredoxin have also been shown to perform the deglutathionylation reaction [35–37]. Currently, there are more than six hundred cellular proteins that have been identified as being glutathionylated [38–40]. Several previous studies have focused on the glutathionylation of cellular proteins in response to viral infection [41,42]; in addition, the viral proteases of human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 1 (HTLV-1) have been shown to be regulated by glutathionylation [43–46]. The two cysteines (Cys67 and Cys95) of HIV-1 protease are highly conserved among isolates from different patients. Moreover, they are not located in the active site, which suggests that these two cysteine residues are not required for proteolytic activity. Nevertheless, they both form mixed disulfides with GSH and give different effects on enzyme activity and inhibit protein dimerization [43,46]. This modification can be reversed by the deglutathionylase activity of endogenous glutaredoxin [45]. In fact, deglutathionylation of Cys95 generates a more active enzyme compared to its fully oxidized form whereas the glutathionylation of Cys67 generates a more active enzyme [45]. Akin to HIV-1, the HTLV-1 protease has also been reported to be controlled by glutathionylation [46]. Davis and colleagues provide evidence that suggests the reversible oxidation of retroviral protease activity is an evolutionarily conserved regulatory mechanism [46].

In this study, we show that the alphavirus chikungunya nsP2 full-length protein can also undergo a reversible glutathionylation modification and that glutathionylation impacts upon the catalytic rates and substrate binding affinities of the protease activity.

2. Materials and methods

2.1. nsP2 recombinant protein expression and purification

The nsP2 full-length protein recombinant clone was expressed and purified as previously described [47]. The purified recombinant proteins were visualized by SDS-PAGE (10% gels). Protein concentration was determined by using Bradford reagent (Bio-Rad Laboratories) with BSA as the standard.

2.2. Analysis of nsP2 glutathionylation by Western blot

The purified nsP2 protein (in 50 mM Tris–HCl pH 8.0) was treated with 5 mM oxidized glutathione (GSSG) at room temperature for 10 min. The treated and untreated samples were separated under non-reduced SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBS-0.1% Tween-20 (PBST) at room temperature for 1 h. Mouse anti-glutathione antibody (US Biological) was used at a 1:1000 dilution overnight at 4 °C. Goat anti-mouse IgG-AP secondary antibody (Santa Cruz) was used at a 1:10,000 dilution at room temperature for 3 h. The signal was detected using the ThermoScientific™ Lumi-Phos WB chemiluminescent substrate. The reversible glutathionylation of nsP2 was assessed by incubation with various concentrations of dithiothreitol (DTT) for 10 min after GSSG treatment.

2.3. Cell culture and virus propagation

The human embryonic kidney cell line (HEK293T/17) was cultured in DMEM supplemented with 10% heat-inactivated FBS and 100 units/ml of penicillin/streptomycin at 37 °C with 5% CO_2. Vero cells were cultured with 5% FBS and 100 units/ml of penicillin/streptomycin.

The CHIKV used in this study was a Thai isolate of an ECSA strain with genotype E1:226V ECSA. The virus was propagated in Vero cells. The virus titers were determined by standard plaque assays as described elsewhere [48].

2.4. Construction of FLAG–nsP2 fusion clone for cell transfection

Specific primers were designed to amplify the full-length protein gene region of nsP2 from our previous recombinant clone [49]. The kozak and FLAG-tag sequence(GACTACAAAGA CGATGACGACAAG) was added to the forward primer. After digestion with restriction enzymes, it was ligated into pcDNA3.1(+) vector. The candidate clones were verified by DNA sequencing.

2.5. Transfection of FLAG–nsP2 and infection with CHIKV

HEK293T/17 cells were seeded on 10 mm tissue culture plates at a density that allowed 80% confluence within 24 h. The cells were transfected with 15 μg of pcDNA-FLAG–nsP2 plasmid using a calcium phosphate transfection method. Briefly, 15 μg of plasmid was diluted in 360 μl sterile water then 360 μl of 2× Hanks buffer saline solution was added to the diluted plasmid. Finally, 36 μl of 2.5 M $CaCl_2$ was added and the mixture was incubated at room temperature for 20 min. After incubation the mixture was added to 10 mm tissue culture plates and the cells were incubated under standard conditions. At 24 h post-transfection (hpi), the cells were infected with CHIKV at MOI of 5 for 2 h. The virus was then removed and the cells were further incubated under standard conditions. The transfected-infected cells were harvested at 0, 6 and 24 hpi. The transfected or infected cells only were harvested at 24 h post-transfection/infection. Mock infection was performed in parallel.

2.6. Determination of enzymatic activity

Enzyme activity assays were performed as previously described [47]. Briefly, the 3 substrates used span the 3 scissile sites of the CHIKV nonstructural polyprotein that are cleaved by nsP2. The three synthetic fluorescent substrates used are AGA (nsP1/nsP2), AGC (nsP2/nsP3) and AGG (nsP3/nsP4). Protease activity was measured at varying substrate concentrations using a Beckman Coulter DTX880 multimode detector at 340 nm excitation and 430 nm emission wavelengths at 37 °C for 3 h. The progress curves were analyzed by Dynafit program© version 3.28.070 (BioKin, Ltd.) to obtain the kinetic parameters [50]. The kinetic parameters were compared between the treated and untreated samples.

2.7. Mass spectrometry determination of glutathionylated cysteines

Purified nsP2 protein was treated with 5 mM GSSG at room temperature for 5 min and then resolved on 10% SDS-PAGE under non-reduced condition. The gel was stained with Coomassie brilliant blue R-250. The nsP2 protein band was excised and sent for LC/MS/MS analysis. The LC/MS/MS was performed by Dr. Sze Siu Kwan at the NTU Mass Spec core facility at the School of Biological Sciences, Nanyang Technological University, Singapore.

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