

Activation of calcium/calmodulin-dependent kinase II following bovine rotavirus enterotoxin NSP4 expression

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ABSTRACT

Objective(s): The rotavirus nonstructural protein 4 (NSP4) is responsible for the increase in cytoplasmic calcium concentration through a phospholipase C-dependent and phospholipase C-independent pathways in infected cells. It is shown that increasing of intracellular calcium concentration in rotavirus infected cells is associated with the activation of some members of protein kinases family such as calcium/calmodulin-dependent kinase II, which plays a crucial role in replication and pathogenesis of the virus. The aim of this study was to expression bovine rotavirus NSP4 gene in HEK293 cell and evaluation of its biological effect related to activation of calcium/calmodulin-dependent kinase II in cell culture.

Materials and Methods: MA104 cells was used as a sensitive cell for propagation of virus and defined as a positive control. The NSP4 gene was amplified and inserted into an expression vector, and introduced as a recombinant plasmid into HEK293T cells. Western blot analysis was performed as a confirmation test for both expression of NSP4 protein and activation of calcium/calmodulin-dependent kinase II.

Results: Expression of NSP4 and activated form of calcium/calmodulin-dependent kinase II were demonstrated by western blotting.

Conclusion: It was shown that the expression of biologically active full-length NSP4 protein in HEK293T cells may be associated with some biological properties such as calcium calmodulin kinase II activation, which was indicator of rotaviruses replication and pathogenesis.

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Introduction

Among abundant protein kinases found in mammalian cell systems, there is a distinct subfamily of serine/threonine-specific protein kinase that are regulated by the Ca⁺⁺/calmodulin complex or other related activators in a calcium concentration dependent manner (1). Members of this family are involved in diverse cellular processes like cell proliferation and death, cell motility, metabolic pathways and autophagy. Calcium calmodulin kinase II (CaMKII) is the member of this family that is involved in several of these cellular processes (2).

Rotaviruses (RV) are the major viral etiologic agent of severe gastroenteritis in young children worldwide affecting approximately 70% of children before the age of 5. These triple-layered viruses contain 11 double-stranded RNA segments. The rotavirus gene 10 segment encodes the nonstructural glycoprotein NSP4. The mechanism of rotavirus induced diarrhea is not fully understood, but it was reported that NSP4 acts

as a viral enterotoxin in induction of diarrhea by causing Ca⁺⁺ influx in the cytoplasm of the infected cell (3-5). The rotavirus NSP4 is a viroporin, as a three-pass transmembrane protein that inserts into the endoplasmic reticulum (ER) (6). This protein is responsible for the mobilization of Ca⁺⁺ intracellular in host cells by two ways: (i) NSP4 synthesized in virus-infected cells releases Ca⁺⁺ by pore formation in the ER membrane or viroporin activity of NSP4, (ii) NSP4 can mobilize Ca⁺⁺ by phospholipase C-dependent pathway (6-8). The existence of two mechanisms to mobilize Ca⁺⁺ has been demonstrated in rotavirus infected cells. Studies have shown that NSP4-induced Ca⁺⁺ mobilization may be responsible for some of the cellular aspects such as calcium-sensitive enzymes. One of these enzymes is CaM kinase II that has an important role in rotavirus replication as well as pathogenesis specially cytoskeleton rearrangement related to calcium pathway (2, 9, 10).

The aim of this study was to examine the entire

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expression of bovine rotavirus NSP4 gene in HEK293, and to evaluate its biological effect related to activation of calcium/calmodulin-dependent kinase II in cell culture model.

Materials and Methods

Cell line and virus infection

The HEK293 T (derived from Human Embryonic Kidney) and MA104 (derived from rhesus monkey kidney) cells line were purchased from the cell bank (Pasteur institute of Iran). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% of heat-inactivated fetal calf serum (FBS), 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml of penicillin, 100 µg/ml of streptomycin and incubated at 37 °C, 5% CO₂.

In this study, the bovine rotavirus strain RF (Invitrogen USA) was used. MA104 cells are sensitive to propagation of the rotavirus. For replication of the virus and as a positive control, MA-104 cells were plated at a density of 10⁵ cells/cm² on sterile glass coverslips, incubated at 37 °C in 5% CO₂ for 24 hr. Bovine rotavirus strain RF was activated with 5 µg/ml of trypsin at 37 °C for 1 hr. MA104 cell monolayers were prewashed with phosphate buffered saline (PBS) and inoculated with activated rotavirus at a multiplicity of infection (MOI) of 10. The inocula were removed 1 hr later and replaced with fresh medium without FBS.

Viral RNA analysis

Rotavirus-infected MA104 cells at 10 hr post infection (hpi) were scraped into the medium, pelleted by low-speed centrifugation, washed once with cold PBS, and then viral RNA was purified from the cells by RNA extraction kit. RNA was analyzed by electrophoresis on 10% polyacrylamide gels, and RNA segmental pattern confirmed by silver staining.

Cloning of the NSP4 gene

The NSP4 cDNA gene was amplified by PCR using specific forward (5'GCG GCC GCA AAG TTC TGT TCC GAG AG3') and reverse (5'CGG CAG CTC AAC CTC TTA CAT CGC TG3') primers, which were aligned with nucleotides 48 to 71 and 735 to 761 of bovine rotavirus NSP4 gene, respectively. The 100 µl reaction mixture contained 9 µl of cDNA, 2 µl (2.5 mM of each dNTPs), 2 µl (10 pmol) of each primer, 10 µl of 10X PCR-buffer, 4 µl (25 mM) MgCl₂, 1 µl (5 unit/ µl) of Taq DNA polymerase and 70 µl of water. Amplification was performed using 30 cycles, with each cycle consisting of a denaturation step at 95 °C for 1 min, a primer annealing step at 55°C for 30 sec, a primer extension step at 72 °C for 1 min and one cycle at 72 °C for 10 min (11). The amplicon was ligated into the unique Apa1 and Xba1 cloning sites of the Eukaryotic expression vector pcDNA3.1(+) (Invitrogen, USA), and transformation was performed

in *Escherichia coli* DH5α cells. Recombinant plasmid was extracted using commercial Kit (Intron, Korea), and the pcDNA containing NSP4 gene was confirmed by double restriction digestion using same Apa1 and Xba1 enzymes. The recombinant plasmid also confirmed by sequencing.

In vitro evaluation of transient expression in HEK293T Cells

In order to monitor the expression of NSP4 recombinant protein, an approximate concentration of 10⁶ HEK293T cells/ml were seeded into a 6-well microtiter plate and incubated overnight in complete medium without any antibiotics. The cells were used for transfection at 80% confluency. The cells were transfected with pcDNA3.1+/NSP4 or pcDNA3.1+ using Lipofectamine 2000 (Invitrogen USA) according to the manufacturer's instruction.

Gel electrophoresis and western blot analysis

Transfected or infected cells were recovered by scraping, washed twice in PBS and lysed for 30 min on ice with Tris-NaCl lysis buffer (0.01 M Tris-HCl, 0.1 M NaCl, 2 mM PMSF, and 1% NP-40), 48 hr post transfection. The lysates were centrifuged (800 g), and the protein was quantitated by Bradford assay and was separated with SDS page. Samples were prepared by heating to 90 °C in SDS sample buffer for 5 min. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in 3% non-fat skimmed milk in TBS plus 0.05% Tween 20 (TBS-T) for 2 hr at room temperature (Abcam, UK). To detect the antigen-antibody complex, the membrane was washed and then incubated with Horseradish Peroxidase (HRP) conjugated anti-rabbit IgG antibody diluted 1:10,000 (Sigma, USA) for 1 hr at room temperature, washed again and followed by developing with diaminobenzidine (Roche, Germany).

Results

Normal RNA migration pattern of bovine rotavirus by silver staining

The viral RNA was purified from the cells, separated by electrophoresis on 10% polyacrylamide gels, and RNA segmental pattern confirmed by silver staining as shown in Figure 1.

Cloning of the NSP4 gene

The NSP4 cDNA gene was amplified by PCR and the amplicon was ligated into the unique Apa1 and Xba1 cloning sites of the Eukaryotic expression vector pcDNA3.1(+). Then the recombinant plasmid pcDNA3.1+/NSP4 was identified by enzyme digestion as shown in Figure 2. The sequencing was confirmed the NSP4 gene fragment with detectable size of 570 bp.



Figure 1. Normal RNA pattern of bovine rotavirus genome 4-2-3-2. The RNAs were extracted from purified virions, separated on a polyacrylamide gel and visualized by silver staining

Gel electrophoresis and western blot analysis

The transfected cell with pcDNA3.1+ /NSP4 along with cells transfected with the empty plasmid pcDNA3.1+ and MA 104 cell infected with rotavirus were analyzed by SDS-PAGE under reducing conditions, which showed a new band with an apparent molecular mass. Taking the molecular weight of the protein (26 kDa), which was expected for the protein as shown in Figure 3.

Rotavirus infection or NSP4 expression leads to activation of calcium calmodulin kinase II

CaM kinase II is a multisubunit enzyme consisting of catalytic, autoregulatory, and subunit assembly domains (12). Upon activation by calcium and calmodulin, CaM kinase II autophosphorylates threonine 286, leading to full activation of the enzyme (13, 14). This phosphorylation allows specific antibodies for threonine 286 to detect the active form of the enzyme. CaM kinase II activation was analyzed by western blotting of crude lysate prepared from control cells and cells transfected with expression plasmid containing rotavirus NSP4 (Figure 4).

Discussion

In the present study, the NSP4 cDNA gene was amplified by RT-PCR and cloned in pcDNA3.1+ expression vector. The recombinant pcDNA3.1+/NSP4 was confirmed and followed by transfection in to HEK293T cells. After that, protein production was evaluated by SDS-PAGE and western blotting. Also, after confirmation of NSP4 expression in the HEK293T cells, the CaM kinase II activation was analyzed by western blotting. The rotavirus enterotoxin NSP4 is a multifunctional protein involved in virus pathogenesis and morphogenesis (3). It has been demonstrated that this protein is a viroporin, which composed of three-pass transmembrane protein that

inserted into the ER and is responsible for the increase in cytoplasmic calcium concentration through a phospholipase C-dependent and phospholipase C-independent pathways (6, 7). Several studies have shown that increase in intracellular calcium concentration in rotavirus infected cells play a crucial role in pathogenesis of virus (15). Jean-Philippe Brunet reported that rotavirus replication induces cytoskeleton alterations of Caco-2 cells and described for microvillar F-actin disassembly. They also reported that rotavirus-induced Ca⁺⁺ increase is responsible for microtubule alteration in differentiated Caco-2 cells (16). The mechanism of rotavirus induced disorganization of cellular cytoskeleton is not fully clear, but it seems that disorganization of cellular cytoskeleton in response to increased level of intracellular calcium is related to expression of NSP4 in rotavirus-infected cells (17, 18). Studies have shown that NSP4-induced Ca⁺⁺ may be responsible for activation of Calcium-sensitive enzymes that are in downstream signaling pathways. One of these downstream signaling pathways is CaM kinase II, which

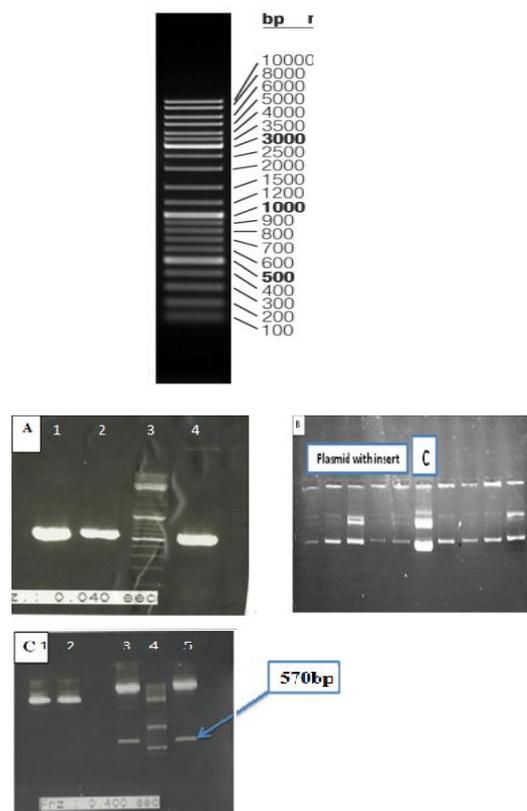


Figure 2. Agarose gel electrophoresis showing amplification and cloning of the NSP4 gene. A; amplification of the NSP4 gene of Bovine rotavirus by RT-PCR, Lane1, 2 and 3, PCR product for complete NSP4 gene (570 bp). Lane 4, size marker (100 bp plus DNA ladder, fermentas); B; Recombinant plasmid extraction. C; Confirmation of recombinant pcDNA NSP4 by double restriction digestion using same Apa1 and Xba1 enzymes

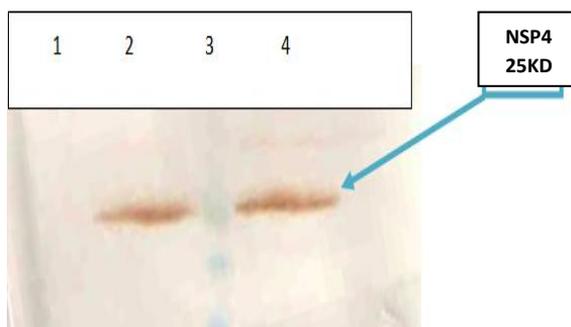


Figure 3. Analysis of NSP4 expression by Western blot analysis. Western blotting carried out using rabbit anti peptide serum diluted 1:200 and HRP-conjugated goat anti-rabbit antibodies. Lane 1, HEK293T cells transfected with empty pcDNA vector as negative control. Lane 2, NSP4 protein (~ 26 KD) expressed in MA104 cells infected by RF rotavirus (8 hpi) as a positive control. Lane 3, NSP4 protein (~ 26 KD) expressed in HEK293T cells transfected with pcDNA. NSP4 vector (48 hr post transfection). Lane 3, molecular weight protein markers (SinaClon, Iran)

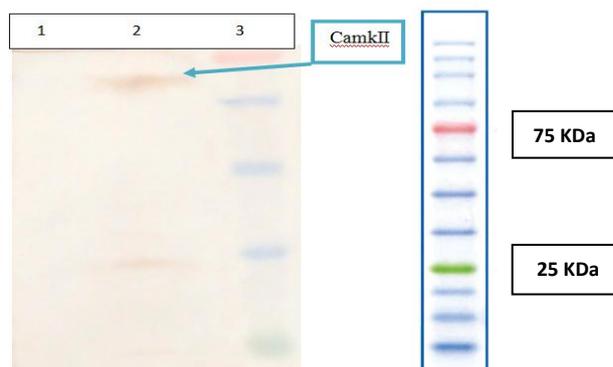


Figure 4. NSP4 mediated activation of CaM kinase II protein detected in HEK293 cells 48 hr post transfection by western blot analysis. Western blotting carried out using rabbit polyclonal antibody diluted 1:1000 and HRP-conjugated goat anti-rabbit antibodies. Lane 1, Mock transfected HEK293 cells as a negative control. Lane 2, activated CaM kinase II as a 60-kDa protein detected in HEK293 cells transfected with lane 3, molecular weight protein markers (11 kDa-245 kDa) (SinaClon, Iran)

has an important role in rotavirus replication and pathogenesis, specially cytoskeleton rearrangement that related to calcium pathway (2, 19-21). Until now, several studies have been performed related to interaction between different viral proteins with cytoskeletal proteins (22, 23). In this context, the CaM kinase II activation was confirmed following African Swine Fever Virus Infection. It was indicated that phosphorylation and rearrangement of vimentin is related to CaM kinase II activation following viral infection (24).

Conclusion

Bovine rotavirus NSP4 gene may be associated with some biological properties such as calcium calmodulin kinase II activation in cell culture models. These biological changes may be related to replication and pathogenesis of the rotavirus. However there is need to more studies in this regard in the future.

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