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Short Report

Effect of Activation and Inhibition of Cellular PKR on Coxsackievirus B3 Replication

Mehdi Zeinoddini[1], Seyyed Mohammad saide Hosseini Amini[1] and Nader Maghsoudi[*1 & 2]

[1]*Dept. of Genetic Engineering, Biotechnology Research Center, Malek Ashtar University;* [2]*Neuroscience Research Center, Shaheed Beheshti University of Medical Science, Tehran, Iran*

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The ds-RNA activated protein kinase (PKR) is a serine-threonine kinase with MW of 68 KDa. It belongs to a family of kinases that control one of the translational initiation factors, eIF2. PKR is produced at high level in response to viral infection. This protein by phosphorylating eIF2 inhibits cellular protein synthesis. In this study, the effect of gamma interferon (IFN-γ), an activator, and 2-aminopurine (2AP), an inhibitor of PKR production on coxsackievirus B3 (CVB3) replication were studied. After addition of IFN-γ and 2AP to Vero and HeLa cultures, cells were infected with CVB3. After 48 h and appearance of cytopathic effect (CPE), cells were collected and viral replication was assessed by standard method. For molecular detection of PKR, RT-PCR technique was used. The data show that interferon inhibited (or lowered) and 2AP promoted viral replication, though in all samples presence of PKR-mRNA could be detected. It seems that PKR plays a key role in CVB3 replication. Therefore, PKR can be considered as a promising and considerable target for designing small molecules and drugs against CVB3. *Iran. Biomed. J. 8 (3): 157-160, 2004*

Keyword: ds-RNA activated protein kinase (PKR), Cytopathic effect (CPE), Coxsackievirus, Interferon-γ

INTRODUCTION

The ds-RNA activated protein kinase (PKR) is a 68-KDa serine-threonine protein kinase, which belongs to the eukaryotic initiation factor-2 α-subunit (eIF-2α) protein kinase family [1, 2]. There are several molecules that promote PKR activity or its inhibities. Amongst the activators of PKR production, interferons are secreted by animal cells in response to viral infection and stress. PKR function is the outcome of interferon response that limits viral protein synthesis in the host cells and is the body's first level of defense against viral infection [3, 4].

In some cases of viral infection, PKR becomes a target for viral-encoded inhibitory molecule, for example in some members of picornavirus family, viral protease disrupts PKR and its inhibitory effect and hence viral replication proceeds [5-7]. These properties of PKR designate it as a promising target for designing small molecules to inhibit viral replication inside host. In this study, the role of PKR on coxsackievirus B3 (CVB3) replication was assessed using gamma interferon (IFN-γ) as an activator and 2-aminopurine (2AP) as an inhibitor [8] of PKR activity. Results revealed a key role for PKR in CVB3 infection and also activity for inhibition of CVB3 replication.

MATERIALS AND METHODS

Cell culture. HeLa and Vero cells were obtained from National Cell Bank of Iran (code number NCBI C115 and NCBI C101). HeLa cells were cultured in a six-well plate in RPMI 1640 with 10% FBS, whereas Vero cells were cultured in a 24-well plate and incubated for 24 h at 37°C.

Virus. CVB3 Nancy strain was obtained from American Type Culture Collection (ATCC number: VR-30). The HeLa and Vero cells were grouped: one batch was incubated with 100 IU/ml of IFN-γ (Immukine, Boehringer Company, Germany) and

*Corresponding Author; Tel: (+98-21) 296 2820; Fax: (+98-21) 295 9493; E-mail: maghsudi@yahoo.com

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the other with 0.05-0.5 mM/ml of 2AP. Negative controls were cells without IFN-γ or 2AP or viruses. Plates were incubated for 1 hour, and then all cultures were infected with 1 ! 1 0^5 PFU of CVB3. Incubation was continued for 48 h for HeLa and 72 h for Vero cells. By observing cytopathic effect (CPE), virus was recovered from the cell suspensions by three cycles of freezing and thawing. Cell debris was pelleted by centrifugation (13,000 ×g, 10 min) and virus in the supernatant was titrated by plaque assay.

Detection of PKR gene. Total RNA of infected HeLa and Vero cells was separately extracted by RNAX kit (Cinagen Company, Iran). Complementary strand DNA (cDNA) was synthesized using random hexamer and reverse transcriptase enzyme and amplified by PCR using primers specific to a conserved region of the PKR gene and then the product was transferred to agarose gel and observed with UV illumination.

Plaque assay. Vero cells (2 ! 10^5 cells/ml) were seeded into 24-well plates and grown overnight in a 37°C incubator with 5% CO_2. The cells were 90 to 100% confluent at the time of infection. Media was aspirated, and 200 µl of serial dilution from virus was added to each well. Infected plates were placed back in the incubator for 1 hour and rocked every 10 minutes. Then, cells were overlaid with 1 ml of 1 × DMEM in 0.5% agar (1:1 mixture of 2 × DMEM at 37°C and 1% agar at 55°C). Cells, 72 hours after infection, were fixed by adding 1 ml of 10% formalin in PBS to each well. After 1 hour, the fixative was poured off and agarose plugs were removed. Cells were stained with 500 µl of 0.5% crystal violet in 20% ethanol per well. Plates were rinsed in tap water and plaques were counted.

RESULTS AND DISCUSSION

The role of PKR in viral host defense and in the antiviral mechanism mediated by interferon is well documented [2, 9]. Several studies have shown that viruses inhibit PKR activity and the loss of this ability is known to result in the loss of resistance to interferon [10]. This fact is easily observed with encephalomyocarditis virus, which is one of the few viruses not to have evolved a mechanism for avoiding activation of PKR [10].

Many studies show that adenovirus synthesizes large quantities of small RNA that inhibits PKR activation [11, 12]. Many other viruses have also developed mechanisms by which inhibit PKR activation [13-21]. In this research, we tried to study the effect of host PKR activation and inhibition on CVB3 replication *in vitro* system. Coxsackieviruses of group B (CVB) are enteroviruses of the picornaviridae family, causative agents of a variety of human diseases, from minor common colds to fatal myocarditis and meningitis [22, 23]. To ensure the presence of PKR within the cells, all the samples (control and infected) were checked for the presence of PKR mRNA using RT-PCR. For a conserved region of PKR cDNA, a set of forward and reverse primers were designed and mRNA was checked using RT-PCR. All hosts were positive for the presence of PKR mRNA in their cytoplasm (Fig. 1).

In the first part of the study, cells were treated with IFN-γ prior to CVB3 infection and checked by PFU (Fig. 2), and compared with control, where no interferon was added prior to infection. Results show that treatment of cells with IFN-γ caused reduction in PFU number from 3.26×10^5 to 2.75×10^5 in HeLa and 1.65×10^5 to 0.066×10^5 in Vero cells (Figs. 3A and B, samples 2 and 3).

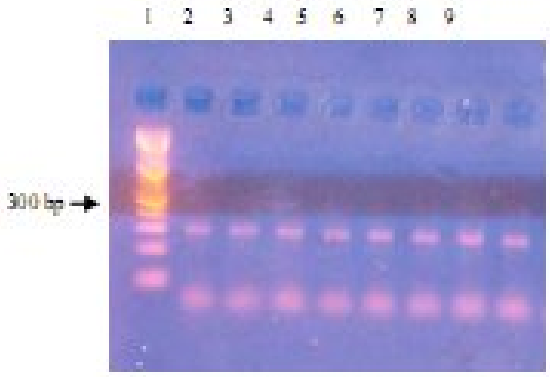

Fig. 1. Analysis of PCR product related to cDNA-PKR on agarose gel. **(1)**, Marker; **(2)**, HeLa, Normal cell, uninfected; **(3)**, HeLa, Normal cell, infected with CVB3; **(4)**, HeLa, treated with IFN-γ (100 IU/ml), infected with CVB3; **(5)**, HeLa, treated with 2AP(0.5 mM/ml), infected with CVB3; **(6)**, Vero, Normal cell, uninfected; **(7)**, Vero, Normal cell, infected with CVB3; **(8)**, Vero, treated with IFN- γ (100 IU/ml), infected with CVB3; **(9)**, Vero, treated with 2Ap(0.5 mM/ml), infected with CVB3.

It is known that the presence of virus ds-RNA in the cytoplasm activates PKR, and consequently the cellular protein synthesis shuts down. In the case of CVB3, treatment of the cells with interferon caused a decrease in PFU. This indicates partial inhibition of viral replication and PKR activation which is

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boosted due to the treatment with interferon, comparing to the control. This was further confirmed when PKR inhibitor was used. In cells treated with 2AP and then infected with CVB3, an increase in viral replication, from 3.26×10^5 to 4.5×10^5 in HeLa and 1.65×10^5 to 9.75×10^5 in Vero cells (Figs. 3A and B, samples 2 and 4) was observed which was indicated by highest PFU value.

These observations justify the role of PKR in CVB3 replication, as activation (using interferon) and inhibition (using 2AP) of PKR affected viral proliferation in host cells comparing to control *in vitro*. Some of the coxsackieviruses are known to interfere with normal host cell translation process by breaking eIF4G (the eukaryotic translation initiation factor 4G) [24] or PABP (poly (A)-binding protein) [25] and turning protein synthesis machinery available for viruses. This mechanism was not assessed in this study, but even if eIF4G or PABP breaking occurs in CVB3 infection, still host PKR plays a key role in CVB3 replication.

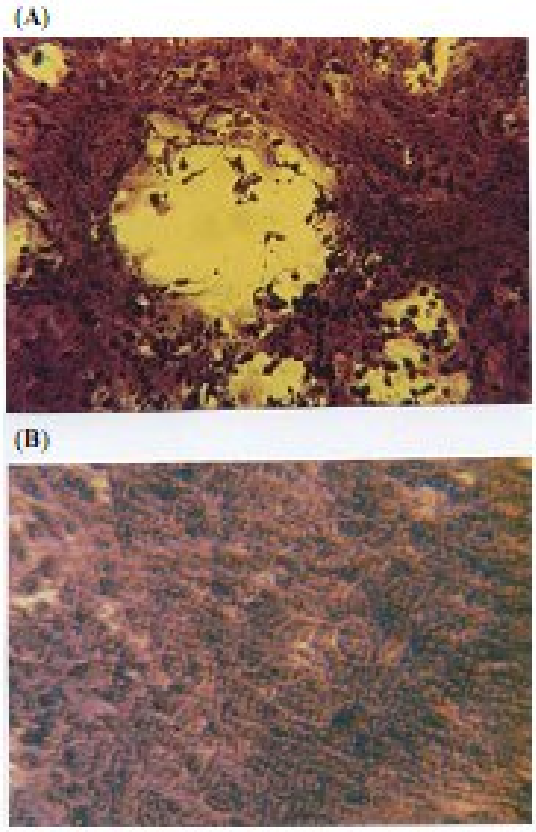

Fig. 2. Plaque of CVB3 in Vero cells. **(A)**, Infected cells; **(B)**, Normal cells.

Characterization of PKR and CVB3 interaction process can help us to design small molecules targeted toward viral proteins, which inhibit PKR activity, with the aim of CVB3 replication control, treatment of CVB3 infection or even prevention of diseases caused by CVB3.

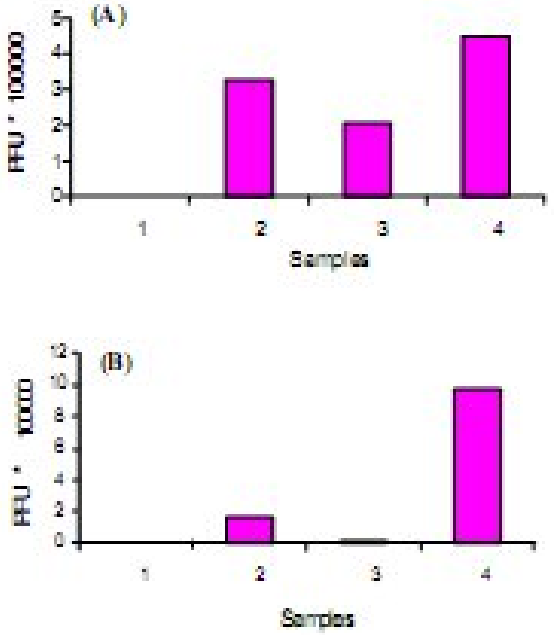

Fig. 3. PFU rate of HeLa **(A)** and Vero cells **(B)** infected by CVB3. A: (1), HeLa, Normal cell, uninfected; (2), HeLa, Normal cell, infected with CVB3; (3), HeLa, treated with IFN-γ (100 IU/ml), infected with CVB3; (4), HeLa, treated with 2AP (0.5 mM/ml), infected with CVB3; B: (1), Vero, Normal cell, uninfected; (2), Vero, Normal cell, infected with CVB3; (3), Vero, treated with IFN-γ (100 IU/ml), infected with CVB3; (4), Vero, treated with 2Ap (0.5 mM/ml), infected with CVB3.

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