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In vitro Interaction of HSV-1 ORF P with Both Thymidine Kinase (TK) and an Unidentified Cellular Protein

A. Karimi[1,*] and B. Soyoofi[2]

[1] *Department of Microbiology and Immunology, Faculty of Medicine, Shahre-Kord University of Medical Sciences, Shahre-Kord, Islamic Republic of Iran*
[2] *Department of Microbiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Islamic Republic of Iran*

Abstract

Herpes simplex virus type-1 (HSV-1) is a neurotropic pathogen of humans that establishes latent infection in the sensory ganglia innervating the site of primary infection. A number of genes control HSV-1 pathogenicity and latency. Open reading frame P (ORF P) is one of these genes that might have a role in latency and pathogenesis. A complication in the analysis of the role of ORF P in the HSV-1 life-cycle is an antisense overlapping gene, ICP34.5. ORF P is also deleted in ICP34.5 negative mutants and to date, no definite function is attributed to it. An approach to analyze the function of a viral gene such as ORF P is to determine if this gene interacts with any of the cellular and viral proteins both *in vitro* and *in vivo*. Therefore, in this work, using GST pulldown assay and Western-blotting**,** it was investigated that with which cellular and viral gene products ORF P interacts. Our results showed that ORF P interacted with Thymidine kinase (TK) and also with an unidentified cellular protein. Conclusively the results of these works together suggest possible role for ORF P in both splicing and replication of HSV-1.

Keywords: Open reading frame (ORF P); Infected cell protein (ICP) 34.5; Thymidine kinase (TK)

Introduction

The Herpesviridae family is double stranded linear DNA enveloped viruses which are widely distributed in nature. These viruses infect mainly vertebrates especially mammals, including human [1]. A number of herpes viruses infect humans as their primary host and cause a wide variety of diseases ranging from minor lesions to viral encephalitis. [2]. Herpes simplex virus (HSV) is one of the most widely distributed in human population and include two types 1 and 2. Herpes simplex virus type-1 (HSV-1) is a neurotropic pathogen of humans that establishes latent infection in the sensory ganglia innervating the site of primary infection [3]. A number of genes include latency associated transcripts (LATs) are involved in virus pathogenicity and latency [3].

LATs arise from the HSV inverted repeats flanking the U_L sequence and therefore present in 2 copies per viral genome [1,2]. The HSV-1 unspliced 8.3 kb LAT has been shown to contain at least 16 open reading frames (ORFs) [4]. One of these ORFs is ORF P which encodes a protein [4-6]. ORF P expressed by HSV-1

* *E-mail: alikarimi72@yahoo.co.uk*

strains (F) and (17^+) is predicted to contain 248 and 233 amino acids, respectively [3]. It is located at the 3′ domain of LAT, almost entirely antisense to and overlaps the ICP34.5 gene and is also contained at 5′ end of the long/short transcripts (L/STs) [4-6]. No definite function has been attributed to [6-9].

ICP34.5 is a HSV-1 neurovirulence gene that is essential *in vivo* and located in the long repeat region of the HSV-1 genome [10].

As most ICP34.5 null mutants affect ORF P due to the extensive overlap of their sequences, it is difficult to assign a role to ORF P [6-9]. An approach to analyze the function of a viral gene such as ORF P is to determine if this gene interacts with any of the cellular and viral proteins both *in vitro* and *in vivo* [11,12]. Therefore, using GST pulldown assay and Western-blotting**,** it was investigated that with which cellular and viral gene products ORF P interacts. Our results showed that ORF P interacted with Thymidine kinase (TK) and also with an unidentified cellular protein.

Materials and Methods

Bacteria

The *E. coli* strain used in this work was BL21 [BF^- dcm ompT hsds (rB-mB-) gal] (Stratagene, UK).

Plasmids

The plasmids used in this work were as follow: i) GST (Pharmacia, UK); GST-ORF P in which ORF P was inserted in frame at the 3′ end of GST.

Cells

Baby hamster kidney 21 clone 13 (BHK) cells [13] were grown in Eagle's medium supplemented with 10% (v/v) newborn calf serum.

Viruses

The wild type virus used was HSV-1 17^+ [14]. The HSV-1 17^+ ICP34.5/ORF P deletion variant 1716 [15] was used as a negative control. *Ts*K, a HSV-1 17^+ mutant with a *ts* lesion in ICP4 [16], which results in overproduction of ORF P at the non-permissive temperature, was also used.

GST Fusion Protein Expression and Purification

A single freshly transformed colony was grown overnight in 5 ml 2YT containing 100 µg/ml ampicillin. This culture was diluted 1/100 in 2YT containing 100 µg/ml ampicillin and grown in a shaking 37°C incubator to reach an OD_{600} of 0.6-0.1. To induce expression of the GST fusion protein, 0.2 mM IPTG was added and the cultures placed back in the incubator for either 2 h at 37°C. After induction, the culture was centrifuged at 13,000 rpm at 4°C for 5 min, the supernatant discarded and the pellet resuspended in 300 µ1 ice cold PBS A. Cells were lysed using a soniprobe and centrifuged at 13,000 rpm for 5 min at 4°C to remove the cell debris. The supernatant was transferred to a fresh eppendorf tube, Sigma USA, 50 µl of 50% (v/v) glutathione agarose beads added to the supernatant and mixed end-over-end for 1-3 h at 4°C. Samples were centrifuged at 13,000 rpm for 1 min at 4°C, the supernatant discarded and 1 ml of PBS A added to the pellet, vortexed, and centrifuged for 1 min at room temperature (RT). Washing was repeated 3 times and the pellet harvested in boiling mixture and analysed by SDS-PAGE or stored at 4°C for use in a pulldown assay.

Pulldown Assay

Freshly prepared glutathione agarose beads, bound to GST fusion proteins, were mixed with 300 or 400 µl labelled (100 µci/ml ^{35}S Metionine) or unlabelled cell protein extract and incubated at 4°C for at least 3 h with continuous end-over-end mixing. The beads were harvested by centrifugation at 13,225 g for 1 min and washed 3 times in 1 ml of an extraction buffer (50 mM NaCl, 0.1% (v/v) NP40, pH 7.5) containing different amounts of NaCl (0.5-500 mM). Again, they were harvested in boiling mixture, stored either at −20°C or boiled for 5 min and analyzed by 12.5% SDS-PAGE. Gels were either fixed, dried and autoradiographed or used for Western-blotting.

Western-Blotting

Samples were separated by 12.5% SDS-PAGE. The proteins in the gel were transferred to the Hybond-ECL membrane, Amersham. After the transfer, the membrane was blocked in PBS containing 0.05% (V/V) Tween 20 (PBS T_{20}) and in 5% (W/V) dried milk at room temperature for 1 h. The primary antibody was incubated either at 37°C or room temperature for 2 h and at 4°C overnight. The membrane was washed 3 times in PBS T_{20} at room temperature for 10 min and then incubated at room temperature for 1 h in the appropriate HRP conjugated secondary antibody (diluted 1:1000 in PBS T/ Marvel) followed by 3 washes in PBS T for 10 min. A chemiluminescence detection reagent (ECL, Sigma, USA) was added to the membrane for 1 min and the membrane was exposed to XS-1 film (Sigma, USA) for the appropriate amount of time.

Results

Expression of GST-ORF P

GST-ORF P should theoretically be about a 55-60 KDa protein. This fusion protein like many HSV-1 proteins is not expressed as full length in bacterial systems [11,17]. During these experiments, we tried to optimize expression of full length GST-ORF P protein. Expression of GST-ORF P was analysed by Western blotting with an anti GST serum (Fig. 1). Expression of GST was similar in both the initial and final experiments (lanes 1 and 3). In the initial experiments, a 40 KDa GST-ORF P doublet was detected in Western blotting (lane 2). In the final experiments the 40 KDa doublet and a higher molecular weight band of 45 KDa was detected (lane 4).

In-vitro *Interaction of ORF P with Thymidine Kinase (TK)*

In an attempt to identify the 45 KDa viral band interacting with GST-ORF P, Western blotting of pulldown extracts was carried out with an antibody against the viral thymidine kinase (TK) [1% (V/V) in PBS/T] [18], which is about 45 KDa (Fig. 2). As expected, TK is detected in 17^+ infected whole cell extract (lane 6) but not in mock whole cell extract (lane 5). The 45 KDa TK band was detected specifically in GST-ORF P pulldowns (lanes 2 and 4) but not in GST pulldown (lanes 1 and 3).

In-vitro *Interaction of ORF P with an Unidentified Cellular Protein*

Using Western blotting of pulldown extracts with an antibody against CKII (β) (1% in PBS/T) [19], an unexpected result was obtained. No specific interaction with CKII (β) was detected. CKII (β) is detected in both 17^+ infected whole extract (Fig. 3, lane 5) and mock infected whole cell extract (lane 8), whereas no specific interaction can be seen in either 17^+ infected (lane 4) or mock infected extracts (lane 7) with GST or GST-ORF P. However, a 45 KDa band is specifically detected in lanes 4 and 7, indicating an unidentified cellular protein, which was also detected by the CKII (β) antibody, interacted with ORF P. As the band detected was slightly lower than that detected in both 17^+ (lane 5) and mock infected cell extracts (lane 8), GST (lane 1) and GST-ORF P (lane 2) extracts were used in Western blotting to rule out cross reaction of the antibody with the GST-ORF P fusion protein. No 45 KDa protein was detected in either of these extracts.

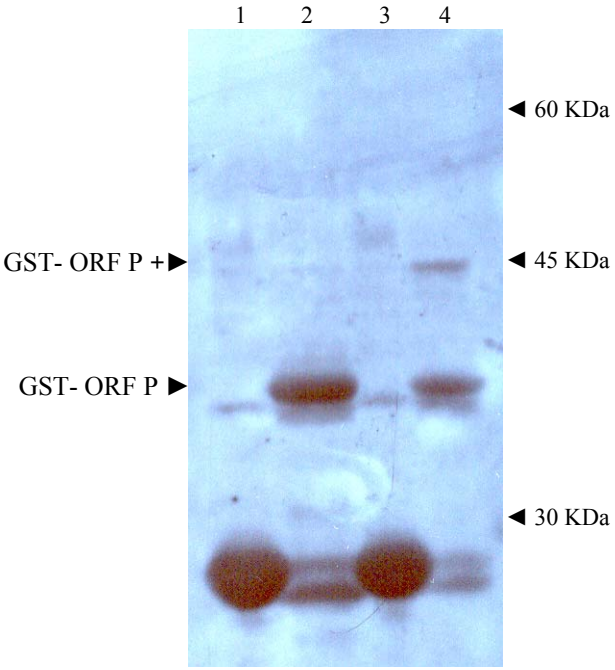

Figure 1. Expression of GST-ORF P. GST-ORF P fusion protein extracts were run on a 10% SDS-PAGE and analysed by Western blotting using an anti-GST serum. Lanes 1 and 3: GST; lanes 2 and 4: GST-ORF P; lanes 1 and 2: initial expression; lanes: 3 and 4: final expression. GST-ORF P related bands 40 KDa (►) and 45 KDa (+►) are indicated. Molecular weights are marked (►).

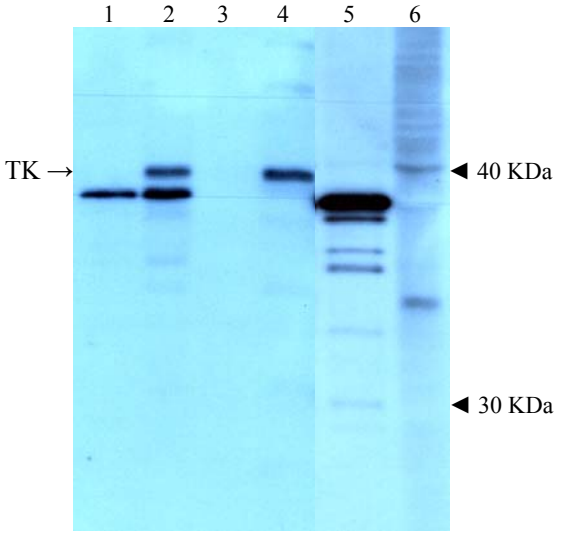

Figure 2. Western blotting of GST-ORFP pull down with TK antibody. GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-TK antibody. Lanes 1, 2 and 6: 17^+ infected cell extracts; lanes 3, 4, and 5: MI extracts; lanes 1 and 3: GST pulldown; lanes 2 and 4: GST-ORF P pulldown; lane 6: whole 17^+ infected cell extracts and lane 5: whole MI cell extract. The TK band (→) is indicated. Molecular weights are marked (►).

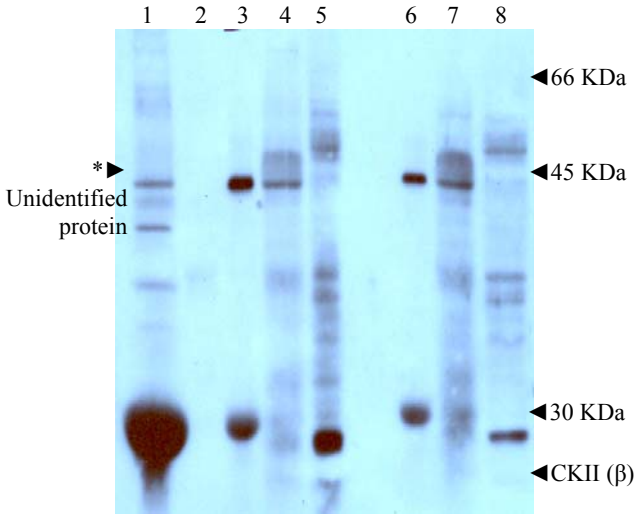

Figure 3. Western blotting of a GST-ORF P pulldown with CKII (β) antibody. GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-CKII (β) antibody. Lane 1: GST; lane 2: GST-ORF P; lane 3-5: 17^+ infected cell extracts; lanes 6-8: MI extract; lanes 3 and 6: GST pulldown; lanes 4 and 7: GST-ORF P pulldown; lane 5: whole 17^+ infected cell extracts and lane 8: whole MI cell extract. CKII (β) (►) and an unidentified protein (*►) are indicated. Molecular weights are marked (►).

Discussion

An alternative way to analyse the function of a protein is to determine those cellular and viral proteins which interact with it. This was carried out for ORF P. There are a number of methods such as GST pulldown, the yeast two-hybrid system and coimmunoprecipitation which can be used to determine protein-protein interactions both *in vitro* and *in vivo.* Using GST-ICP27 and GST-ICP34.5, it was previously shown that GST pulldown is useful for determining protein-protein interactions *in vitro* [11,20] thus this method was used in this work to determine the interaction of ORF P with cellular and viral proteins.

We screened out our GST-ORF P pulldowns with a range of antibodies against proteins with a role in posttranscriptional regulation and which were of an approximate molecular weight to those previously identified (personal data). Western blotting of GST-ORF P pulldowns was carried out with a number of antibodies. Using an antibody against CKII (β), an unexpected result was obtained. No specific interaction of GST-ORF P with CKII (β) was detected. However, a 45 KDa band was detected both in cell extracts and from the GST pulldown, indicating an unidentified cellular protein was interacting with ORF P. There are some previous evidences indicating that ORF P interacts with a number of splicing factors and may play a role in splicing [21,22]. As the size of this cellular protein is in the range of splicing factors [22], it could be one on of the above-mentioned factors.

To identify the 45 kDa viral band interacting with GST-ORF P in pulldown assay, Western blotting of pulldown extracts was carried out with an antibody against HSV-1 TK, which is about 45 KDa [23]. Our results showed that a 45 KDa band was specifically detected, indicating interaction of ORF P with TK.

Although TK is not essential for replication of HSV in tissue culture, it is involved in nucleotide metabolism, in viral virulence [24] and in antiherpetic therapy [25,26].

Previously it was shown that ORF P interacts with a number of splicing factors such as splicing membrane (SM) and P32 [21]. It was also shown that ORF P interacts with SC35 *in vitro* and colocalizes with it in the nucleus of infected cells [27]. The result of this work indicates the interaction of ORF P with Tk. Conclusively, the results of present study, together, suggest possible role for ORF P in both splicing and replication of HSV-1. However, further works are needed to determine if ORF P interacts with any of the proteins *in vivo*.

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