

Expression and *in vitro* Characterization of Herpes Simplex Virus 1 (HSV-1) ORF P Protein

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ABSTRACT

Herpes simplex virus 1 (HSV-1) unspliced 8.3 latency associated transcript (LAT), which located in the long repeat sequences, has been shown to contain at least 16 open reading frames (ORF: A-P). One of these ORF, ORF P, maps almost entirely antisense to HSV-1 neurovirulence gene, ICP34.5. Both ORF P and ICP34.5 are located in the long repeat and are antisense overlapping genes. Therefore, in ORF P deletion mutants, ICP34.5 is also deleted and thus, the characterization of ORF P mutants is almost impossible. An alternative way to analyze its function is to determine those cellular and viral proteins which interact with ORF P. During these experiments, firstly, the expression of full length Glutathione-S-transferase (GST)-ORF P fusion protein was optimized and then, using GST-pull down, it was shown that ORF P interacts with a viral and a few cellular proteins *in vitro*. Conclusively, ORF P might have some functions in HSV-1 replication cycle. *Iran. Biomed. J. 10 (1): 9-13, 2006*

Keywords: Open reading frames (ORF) P, ICP34.5, Herpes simplex virus 1 (HSV-1)

INTRODUCTION

Herpes simplex virus 1 (HSV-1) unspliced 8.3 latency associated transcript (LAT), which located in the long repeat sequences, has been shown to contain at least 16 open reading frames (ORF: A-P) [1]. One of these ORF, ORF P, maps almost entirely antisense to HSV-1 neurovirulence gene, ICP34.5 [1, 2]. Detailed analysis of the products encoded by ORF P showed that ORF P expresses a 248-amino acid protein in strain F and a 233-amino acid protein in strain 17⁺[1]. Both ORF P and ICP34.5 are located in the long repeat and are antisense overlapping genes [3]. Therefore, in ORF P deletion mutants, ICP34.5 is also deleted and thus, the characterization of ORF P mutant is almost impossible. To date, no definite role has been attributed to ORF P except a possible role in splicing at early times in infection [4].

An alternative way to analyze its function is to determine those cellular and viral proteins which interact with ORF P. The Glutathione-S-transferase (GST) pull down is a well characterized method and it was previously shown that it is useful for determining protein-protein interactions *in vitro* [5,

6]. Therefore, it was used in this work to determine the interaction of ORF P with cellular and viral proteins. ORF P protein, like many HSV-1 proteins, is not expressed in full length in the bacterial system [7]. During these experiments, firstly, the expression of full length GST-ORF P protein was optimized and then, using GST-pull down, it was shown that ORF P interacts with a viral and a few cellular proteins *in vitro*.

MATERIALS AND METHODS

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

Bacterial culture media. The bacterial strain was grown in L-broth (170 mM NaCl, 10 g/L bacto-peptone, 5 g/L yeast extract) or 2YT broth (85 mM NaCl, 16 g/L bacto-peptone, 10 g/L yeast extract). L-broth/agar plates were made with 1.5% (w/v) agar in L-broth. Where necessary, media and L-broth/agar plates were supplemented with the appropriate antibiotic, e.g. 100 µg/ml ampicillin.

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

Transformation of bacterial cells. L-broth or 2YT broth (5 ml) were inoculated with *E. coli* and grown overnight in a shaking incubator at 37°C. Fresh media (100 ml) were inoculated with 1 ml of the overnight culture and grown for approximately 3 h until an OD₆₀₀ of 0.6-1.0 had been reached. The cells were pelleted by centrifugation at 500 ×g at 4°C for 15 min and the bacterial pellet resuspended in 50 ml of 50 mM CaCl₂. The bacteria were pelleted as before, resuspended in 10 ml of 50 mM CaCl₂ and incubated on ice for 30 min-24 h and used for transformation.

Expression and purification of GST fusion protein. A single freshly transformed colony was grown overnight in 5 ml 2YT broth containing 100 µg/ml ampicillin. This culture was diluted 1/100 in 2YT containing 100 µg/ml ampicillin and grown in a shaking incubator at 37°C to reach an OD₆₀₀ of 0.6-0.1. To induce expression of the GST fusion protein, 0.2 mM Isopropyl-B-D-thiogalactoside (IPTG) was added and the cultures placed back in the incubator for either at 37°C for 2 h. After induction, the culture was centrifuged at 19,000 ×g at 4°C for 5 min, the supernatant discarded and the pellet resuspended in 300 µl ice cold PBS (phosphate buffer saline). Cells were lysed using a soniprobe and centrifuged at 19,000 ×g at 4°C for 5 min to remove the cell debris. The supernatant was transferred into a fresh Eppendorf tube, 50 µl of 50% (v/v) glutathione agarose beads added to the supernatant and mixed end-over-end at 4°C for 1-3 h. Samples were centrifuged at 19,000 ×g at 4°C for 1 min, the supernatant discarded and 1 ml of PBS added to the pellet, vortexed, and centrifuged for 1 min at room temperature. Washing was repeated 3 times and the pellet harvested in boiling mixture and analyzed by SDS-PAGE or stored at 4°C for use in a pull down assay.

Cell extract preparation for GST pull down. For pull down assays, soluble protein extracts were prepared from either labelled or unlabelled infected or mock infected (MI) cell extracts. For labelled extracts, proteins were labelled with 100 µCi/ml [³⁵S]-methionine in E-met/5. Cell monolayers were washed twice with PBS, 1.3 ml 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) extraction

buffer added for 30-60 min at 4°C and cells harvested by scraping. The cells were lysed by sonication for 2 min in a sonibath and cell debris pelleted by centrifugation at 19,000 ×g for 2 min. The pellet was discarded and soluble protein extracts were stored at -70°C.

GST-pull down assay. Freshly prepared glutathione agarose beads with bound GST fusion proteins were mixed with 300 or 400 µl labelled 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 19,000 ×g for 1 min and washed 3 times in 1 ml HEPES extraction buffer containing different amounts of NaCl (0.5-500mM). The beads were harvested in boiling mixture and either stored at -20°C or boiled for 5 min, analyzed by SDS-PAGE gels were either fixed, dried and autoradiographed or used for Western-blotting.

RESULTS

Expression and optimization of GST-ORF P. To express a protein in *E. coli*, GST is commonly used as a fusion partner [8]. During the expression of the fusion protein, several factors such as temperature, time, IPTG and *E. coli* strain can be varied to maximize the amount, length, and solubility of the protein. The expression of GST-ORFP in a bacterial system had not been previously optimized. Thus, using a number of *E. coli* strains at different temperatures and different amounts of IPTG, expression of the amount and length of GST-ORF P was optimized (data not shown). Eventually, at the final experiment (Fig. 1), a full length of GST-ORF P which is 66 kDa was obtained (lane 1). GST was used as a control and its full length is 28 kDa (lane 2).

Interaction of HSV-1 ORF P with a number of cellular and a viral protein by using pull down assay. GST fusion protein in which the protein of interest is cloned in frame with the carboxy terminus of GST is widely used to identify new protein: protein interactions or to confirm and investigate those identified in other systems [9]. The concentration of salt, detergents and both target and interacting proteins affect interaction of the two proteins in pulled down assay. The standard concentration of NaCl in washing buffer is 0.5 mM.

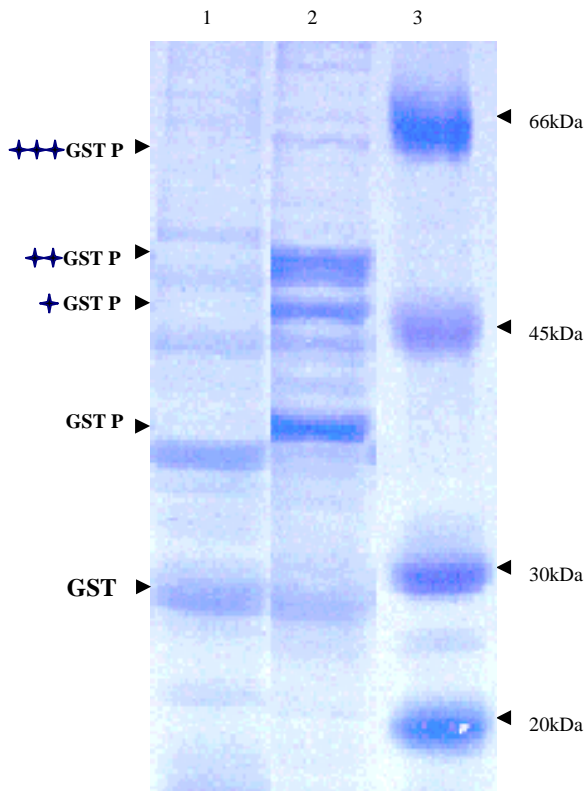


Fig. 1. Expression of GST, GST-ORF P at final experiment. GST pull down extracts were run on a 5-12.5% gradient SDS-PAGE and analyzed by Coomassie blue staining. Lane 1, GST; lane 2, GST-ORF P; lane 3, molecular weight marker with sizes shown. GST-ORF P related bands of 40 kDa (.....), 45 kDa (+), 50 kDa (++) and 66 kDa (+++) are indicated.

As usual, washing with 0.5 mM NaCl containing wash buffer resulted in no specific bands being detected. (data not shown). By increasing the salt concentration, the stringency of the washing buffer would increase and hopefully remove non-specific binding bands leaving specific interacting bands visible. Thus, there was an attempt to optimize the detection of interacting bands by GST/ORF P by changing the salt concentration of the washing buffer. Using 5 mM NaCl containing wash buffer (personal data), no specific bands were still detected. However, increasing the NaCl to 50 mM (Fig. 2) resulted in a number of specific bands being detected. These bands include 6 cellular and one viral protein with sizes of 24, 26, 27, 33, 40 (viral), 47, and 60 kDa (lanes 2 and 4).

DISCUSSION

HSV-1 ORF P overlaps and is antisense to ICP34.5 gene. An alternative way to analyze

function of a gene is to determine those cellular and viral proteins which interact with it. This was carried out for ORF P. It was previously shown that GST pull down is useful for determining protein-protein interactions *in vitro* [9, 10] and thus this method was used in this work to determine the interaction of ORF P with cellular and viral proteins.

The main problem faced in carrying out the pull down experiments was the lack of expression of full length GST-ORF P fusion protein. This fusion protein like many HSV-1 proteins does not express as full length in bacterial systems [11, 12]. Using a number of *E. coli* strains and different temperatures

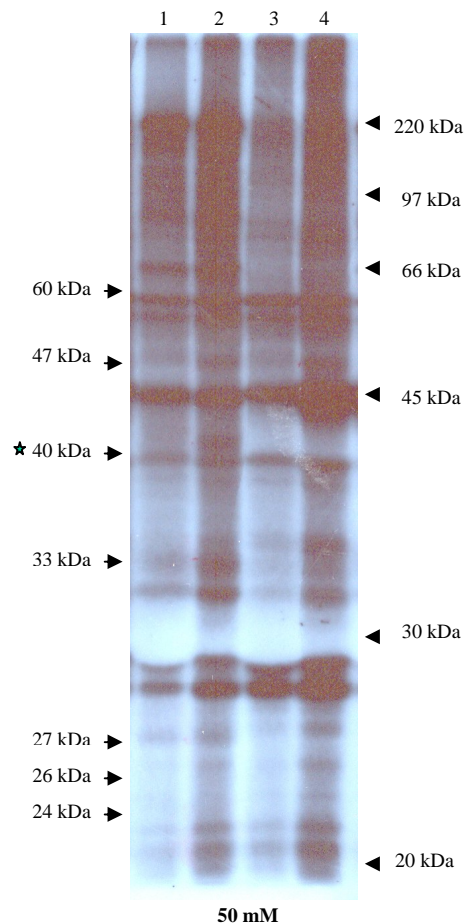


Fig. 2. Optimization of GST pull down experiment by the use of different concentration of NaCl in the washing buffer. A GST pull down was performed on [³⁵S] methionine labelled 17+ infected (lanes 1 and 2) and mock infected (MI) cell extracts (lanes 3 and 4), analyzed by SDS-PAGE and autoradiography. Pull downs were washed with 50 mM NaCl. Lanes 1 and 2, 17+ infected extract; lanes 3 and 4, MI extracts; lanes 1 and 3, GST; lanes 2 and 4, GST-ORF P. GST-ORF P interacting cellular (▶) and viral (★▶) bands with their molecular weights are indicated. Molecular weight is marked.

of induction, the expression of GST-ORF P in terms of both amount and length was optimized. In the final experiments, two higher molecular weight bands of 50 and 60 kDa were detected. GST-ORF P should theoretically be about a 55-60 kDa protein [1, 13, 14]. Thus, as the results showed, at the end, some nearly full length GST-ORF P was expressed and this preparation was used for the pull down assays.

In the initial experiments, using the standard conditions for a GST pull down assay, no ORF P interacting proteins were detected. This method had previously identified a number of cellular proteins interacting with GST-ICP27 [6, 10, 15] and GST-ICP34.5 [16, 9]. As with co-immunoprecipitation, salt and detergent concentrations in the buffers along with target and interacting protein concentrations affect which proteins are pulled down. By changing the salt concentration of the washing buffer, the detection of proteins interacting with GST-ORF P was optimized. The standard buffer used contained 0.5 mM NaCl and by increasing the salt concentration it would increase the stringency of the washing buffer and hopefully remove non-specific binding proteins leaving specific interacting proteins visible. In the initial experiments, using the standard conditions for a GST pull down assay, no ORF P interacting proteins were detected. Therefore, to optimize this method, washing with 0.5 and 5 mM NaCl containing wash buffer resulted in no specifically bound proteins being detected (data not shown). However, increasing the NaCl to 50 mM resulted in binding by a number of specific proteins being detected. These include one viral and 6 cellular proteins. ORF P N-terminal was cloned (in C-terminal of GST) as a 3' GST fusion protein [9] and thus is intact. As different lengths of GST-ORF P fusion protein were expressed (mostly truncated), all the fusion proteins will include the amino-terminus but be truncated at the carboxy-terminus. Thus, the proteins identified might interact with the amino-terminus of ORF P.

There is some previous evidence indicating that ORF P interacts with a number of splicing factors and may play a role in splicing [4, 17]. Using GST pull down assay, the result of this work also showed that ORF P interacts with a number of cellular proteins and one viral protein (Fig. 2). Based on this method, as a number of specific interactions detected in both viral and MI cell extracts with GST-ORF P (and not with GST) (lanes 2 and 4), these bands are cellular proteins interacted with ORF P. Also, as one specific interaction detected in

only viral infected cell extracts (not in mock extract) with GST-ORF P (lane 2), this band is a viral protein. Conclusively, these results indicate that ORF P might have a role in HSV-1 replication cycle which has still to be determined.

REFERENCES

1. Lagunoff, M. and Roizman, B. (1994) Expression of a herpes simplex virus 1 open reading frame antisense to the γ (1)34.5 gene and transcribed by an RNA 3' coterminal with the unspliced latency-associated transcript. *J. Virol.* 68: 6021-6028.
2. Randall, G. and Roizman, B. (1997) Transcription of the derepressed open reading frame P of herpes simplex virus 1 precludes the expression of the antisense gamma (1)34.5 gene and may account for the attenuation of the mutant virus. *J. Virol.* 71: 7750-7757.
3. Ward, S.L., Scheunder, D., Poppers, J., Kaufman, R.J. and Leib, D.A. (2003) *In vivo* replication of an ICP34.5 second-site suppressor mutant following corneal infection correlates with *in vitro* regulation of eIF2 alpha phosphorylation. *J. Virol.* 77: 4626-4634.
4. Bruni, R. and Roizman, B. (1996) Open reading frame P of herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mRNA. *Proc. Natl. Acad. Sci.* 93: 10423-10427.
5. Brown, S.M., MacLean, A.R., McKie, E.A. and Harland, J. (1997) The herpes simplex virus virulence factor ICP34.5 and the cellular protein MyD116 complex with proliferating cell nuclear antigen through the 63-amino-acid domain conserved in ICP34.5, MyD116, and GADD34. *J. Virol.* 71: 9442-9449.
6. Bryant, H., Wadd, S., Filhol, O., Scott, J.E., Hsieh, T.Y., Everett, R.D. and Clements, J.B. (1999) The multifunctional herpes simplex virus IE63 protein interacts with heterogeneous ribonucleoprotein K and with casein kinase 2. *J. Biol. Chem.* 274: 28991-28998.
7. Cheng, G., Gross, M., Brett, M.E. and He, B. (2001) AlaArg motif in the carboxyl terminus of the gamma γ (1) 34.5 protein of herpes simplex virus type 1 is required for the formation of a high-molecular-weight complex that dephosphorylates eIF-2 alpha. *J. Virol.* 75: 3666-3674.
8. Smith, D.B. and Johnson, K.S. (1998) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67: 31-40.
9. Brown, S.M., MacLean, A.R., McKie, E.A. and Harland, J. (1997) The herpes simplex virus virulence factor ICP34.5 and the cellular protein

- MyD116 complex with proliferating cell nuclear antigen through the 63-amino-acid domain conserved in ICP34.5, MyD116, and GADD34. *J. Virol.* 71: 9442-9449.
10. Bryant, H.E., Matthews, D.A., Wadd, S., Scott, J.E., Kean, J., Graham, S., Russell, W.C. and Clements, J.B. (2000) Interaction between herpes simplex virus type 1 IE63 protein and cellular protein p32. *J. Virol.* 74: 11322-11328.
 11. Bryant, H.E., Wadd, S.E., Lamond, A.I., Silverstein, S.J. and Clements, J.B. (2001) Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. *J. Virol.* 75: 4376-4385.
 12. Lagunoff, M. and Roizman, B. (1995) The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus 1 genome. *J. Virol.* 69: 3615-3623.
 13. Hagglund, R., Munger, J., Poon, A.P., Roizman, B. (2002) U(S)3 protein kinase of herpes simplex virus 1 blocks caspase 3 activation induced by the products of U(S)1.5 and U(L)13 genes and modulates expression of transduced U(S)1.5 open reading frame in a cell type-specific manner. *J. Virol.* 76 (2): 743-754.
 14. Randall, G., Lagunoff, M. and Roizman, B. (2000) Herpes simplex virus 1 open reading frames O and P are not necessary for establishment of latent infection in mice. *J. Virol.* 74: 9019-9027.
 15. Chen, S.H. and Lee, L.Y. (2002) Neither LAT nor open reading frame P mutations increase expression of spliced or intron-containing ICP0 transcripts in mouse ganglia latently infected with herpes simplex virus. *J. Virol.* 76: 4764-4772.
 16. Lee, L.Y. and Schaffer, P.A. (1998) A virus with a mutation in the ICP4-binding site in the L/ST promoter of herpes simplex virus type 1, but not a virus with a mutation in open reading frame P, exhibits cell-type-specific expression of gamma (1)34.5 transcripts and latency-associated transcripts. *J. Virol.* 72: 4250-4264.
 17. Ward, P.L., Taddeo, B., Markavitz, N.S., Roizman, B. (2000) Identification of a novel expressed open reading frame situated between genes U(L)20 and U(L)21 of the herpes simplex virus 1 genome. *Virology* 20: 275-285.