Identification and characterization of the herpes simplex virus type 1 UL51 gene product

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The products of the herpes simplex virus type 1 UL51 gene were identified as phosphoproteins with apparent molecular masses of 27, 29 and 30 kDa. The proteins were produced at the late stage of infection, in a manner highly dependent on viral DNA synthesis, and were associated with extra-cellular virions. Immunofluorescence studies localized the UL51 proteins mainly to the cytoplasm, both in infected Vero cells and in transfected COS-1 cells singly expressing the UL51 gene.

Herpes simplex virus (HSV) is a large, enveloped DNA virus and the genome contains at least 84 different genes (Roizman et al., 1996). Approximately half of these genes have been shown to be dispensable for virus replication in cell cultures, but the proteins encoded by these dispensable genes are considered to play important roles in various aspects of virus growth and spread in the host. Although recent studies have identified most of the dispensable genes of HSV (Roizman & Sears, 1996; Roizman et al., 1996), the precise functions of many gene products remain obscure. The UL51 gene of HSV-1 is one of the dispensable genes that are located at the right end of the unique long (UL) region of the virus genome (Barker & Roizman, 1990; McGeoch et al., 1988) and its gene homologues are conserved throughout the herpesvirus family (Albrecht et al., 1992; Baer et al., 1984; Baumeister et al., 1995; Chee et al., 1990; Davison & Scott, 1986; Gompels et al., 1995; Telford et al., 1992). The HSV-1 UL51 gene is predicted to encode a protein of 244 amino acids and its homologues have similar molecular size. Recently, Lenk et al. (1997) identified the UL51 product of pseudorabies virus (PRV), one of the alphaherpesviruses, and demonstrated that the 30 kDa product is a virion component and mainly localized in the nuclei of infected cells. To our knowledge, however, there are no reports concerning the UL51 protein of HSV, although defects in the gene have been shown to be associated with a small plaque phenotype (Barker & Roizman, 1990). The present study was undertaken to identify and characterize the product of the HSV-1 UL51 gene.

To identify the UL51 protein, we first generated anti-UL51 rabbit antisera by using a recombinant HSV-1 UL51 fusion protein as antigen. Plasmid pET28-UL51 was constructed for this purpose, as described previously (Daikoku et al., 1997; Yamada et al., 1997). Briefly, the UL51 coding sequence was cloned by PCR amplification from HSV-1 KOS genomic DNA, with UL51f (5′ AACGTCGACTTATTGACCCAAAACAC-CAC) as the forward primer and UL51r (5′ CGGAATTCTGTCTCTTTCG) as the reverse primer. Sall and EcoRI sites were incorporated into the forward and reverse primers, respectively, to facilitate cloning. The PCR product was digested with Sall and EcoRI and then cloned in-frame and downstream of the region encoding the initiating ATG plus six histidine (6×His) residues in the E. coli expression vector pET28a (Novagen), to give pET28-UL51. The UL51 fusion protein, with an apparent molecular mass of 32 kDa, was then expressed in E. coli following treatment with IPTG (Fig. 1A), purified with the Prep Cell system (Bio-Rad), and the purified fractions were used to immunize three rabbits as described previously (Daikoku et al., 1997). The reactivity and specificity of the antisera were examined by Western blotting (Fig. 1B). One of the antisera reacted strongly to proteins with molecular masses of 27 and 29 kDa in the lysate of HSV-1-infected Vero cells. This antiserum also reacted to proteins with molecular masses of 28 and 30 kDa in HSV-2-infected cell lysates, and these protein bands could not be detected in mock-infected cell lysates (Fig. 1B, lane 4). The reactivity of the antiserum to these proteins was clearly eliminated by pre-adsorption of the antiserum with a lysate from control E. coli (Fig. 1B, lane 7) but there was no marked change in the reactivity to these protein bands after pre-adsorption with a lysate from control E. coli (Fig. 1B, lanes 7 and 8). Preimmune rabbit serum did not react to any specific proteins in HSV-1- or HSV-2-infected cells (Fig. 1B, lanes 2 and 3). These results indicate that the antiserum could detect the UL51 protein specifically in HSV-1- and HSV-2-infected cells; we therefore used this polyclonal antiserum for further experiments to characterize the HSV-1 UL51 gene product.

The time-course of UL51 protein synthesis in HSV-1-infected Vero cells was analysed by Western blotting. At
Fig. 1. (A) Induction of His-tagged UL51 fusion protein in E. coli. E. coli cells harbouring pET28-UL51 were grown in the absence (lane 1) or presence (lane 2) of IPTG, and the UL51 fusion protein was purified with the Prep Cell system (Bio-Rad) (lane 3). Proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue. (B) Reactivity and specificity of rabbit polyclonal antiserum against His-tagged UL51 fusion protein. Vero cells were mock-infected (lanes 1 and 4) or infected with HSV-1 (lanes 2, 5, 7 and 9) or HSV-2 (lanes 3, 6, 8 and 10), and harvested at 15 h p.i. Proteins were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with preimmune serum (lanes 1–3), anti-UL51 serum (lanes 4–6), anti-UL51 serum pre-adsorbed with a lysate from control E. coli (lanes 7 and 8) or anti-UL51 serum pre-adsorbed with a lysate from E. coli expressing the UL51 fusion protein (lanes 9 and 10). (C) Production of UL51 protein in HSV-1-infected cells. Vero cells were mock-infected (lane 1) or infected with HSV-1 (lanes 2–8) at a multiplicity of 5 p.f.u. per cell. The cells were cultured in the absence (lanes 1–7) or presence (lane 8) of 300 mg/ml ACV and harvested at 3 (lane 2), 6 (lane 3), 9 (lane 4), 12 (lane 5), 18 (lane 6) or 24 (lanes 1, 7 and 8) h p.i. Proteins were separated by SDS–PAGE and analysed by Western blotting with anti-UL51 serum. (D) Detection of UL51 protein by immunoprecipitation. Vero cells were mock-infected (lanes 1, 6, 8 and 11) or infected with HSV-1 (lanes 2–5, 7, 9, 10 and 12) at a multiplicity of 5 p.f.u. per cell. At 8 h p.i. the cells were pulse-labelled with 100 mCi/ml [35S]methionine (lanes 1–7) or with 100 mCi/ml [32P]orthophosphate (lanes 8–12) for 10 min, and then chased for 0 (lanes 1, 2, 8 and 9), 30 (lane 3), 60 (lanes 4, 6, 7, 10–12) or 120 (lane 5) min. Immunoprecipitation with the anti-UL51 serum (lanes 1–5, 8–10) or with preimmune serum (lanes 6, 7, 11 and 12) was performed as described previously (Yamashita et al., 1996a). In all panels, arrowheads indicate the UL51 gene products and the positions of molecular mass markers are shown to the left.

Various times after infection cell lysates were subjected to electrophoresis, transferred to PVDF membranes (Immobilon, Millipore) and reacted with the UL51 antiserum. As shown in Fig. 1(C), UL51 protein was first detectable as a single protein band of 27 kDa at 6 h post-infection (p.i.). The 29 kDa species was detected at 9 h p.i., and thereafter these proteins gradually increased in amount until 18 h p.i. A 30 kDa species was also detectable in this blot as a faint band. These results indicate that the UL51 protein is a late gene product. To determine the dependence of its production on viral DNA synthesis, infected cells were maintained for various times after a 1 h adsorption period in the presence of 300 mg/ml acyclovir (ACV). UL51 protein production was not detectable in the presence of ACV, even at 24 h p.i. (Fig. 1C, lane 8), indicating that UL51 protein synthesis was highly dependent on viral DNA synthesis. These results suggest that UL51 was regulated as a γ2 gene, but Northern blot analysis would be required to confirm this.

In an attempt to explain the presence of two or three bands associated with the UL51 protein, we examined whether the UL51 protein was modified by phosphorylation. HSV-1-infected cells were pulse-labelled with [35S]methionine or [32P]orthophosphate for 10 min at 8 h p.i., chased for the times indicated in the figure legend and analysed by immunoprecipitation using UL51 antiserum (Fig. 1D). The 27 kDa species was readily detected when HSV-1-infected, [35S]methionine-labelled cells were lysed immediately after the pulse and subjected to immunoprecipitation; after a 60 min chase the 29 and 30 kDa proteins were also easily detectable.
Fig. 2. Localization of UL51 protein in HSV-1-infected cells. (A)–(D) Mock-infected (A) and HSV-1-infected Vero cells at 9 (B, D) and 12 (C) h p.i. were fixed with cold acetone and stained with anti-UL51 serum (A–C) or preimmune serum (D). (E)–(F) Indirect immunofluorescence of Vero cells expressing the UL51 protein alone. Semi-confluent monolayers of Vero cells on glass cover slips in 35 mm plastic dishes were washed with PBS and overlaid with 1 ml Dulbecco’s modified Eagle’s minimal essential medium containing 10 µl lipofectin reagent (Gibco BRL) and 3 mg pcDNA-UL51. Cells were incubated at 37 °C for 6 h and the medium was replaced with 2 ml Eagle’s minimal essential medium containing 10% foetal calf serum. Following incubation at 37 °C for 24 (E) or 48 (F) h, cells were fixed and treated with anti-UL51 serum for indirect immunofluorescence staining.

$^{32}$P-labelled proteins with the same apparent molecular masses were immunoprecipitated by the UL51 antiserum from HSV-1-infected cell lysates. None of these proteins was immunoprecipitated either with preimmune antiserum or from mock-infected cells. These results suggest that the UL51 protein was phosphorylated in infected cells. However, it remains unclear whether the generation of the 29 and 30 kDa species was caused only by modification by phosphorylation.

The intracellular distribution of UL51 protein was examined by indirect immunofluorescence staining. At various times after infection, Vero cells infected with HSV-1 were fixed with cold acetone, treated with human serum to block nonspecific binding and reacted with the UL51 antiserum. Specific fluorescence became detectable in the cytoplasm of infected cells at 6 h p.i. At 9 h p.i., UL51-specific fluorescence was found distributed in the cytoplasm and the perinuclear region; a typical pattern of staining is shown in Fig. 2(B). This pattern of cytoplasmic staining continued throughout the course of infection, although at later times of infection some infected cells also contained diffuse fluorescence over the nucleus (Fig. 2 C). No specific fluorescence could be detected with the UL51 antiserum in mock-infected cells (Fig. 2 A). However, some fluorescence was detectable in the perinuclear region at the late stage of infection when infected cells were incubated with preimmune serum (Fig. 2 D).

To determine whether the UL51 protein was a component of HSV-1 virions, extracellular virions were collected from culture media harvested at 36 h p.i. Virus particles were pelleted by centrifugation at 87,000 g for 1 h and purified by sucrose density-gradient centrifugation. Fractions were col-
selected from the bottom to the top of the gradient and the protein composition of each fraction was analysed by SDS–PAGE followed by silver staining. As shown in Fig. 3(A), HSV virions were detected as a peak in fractions 8–10. When fractions were subjected to Western blot analysis with the UL51 antiserum, the 27 and 29 kDa proteins were detected in the fractions corresponding to the peak of HSV virions. These results suggest that the UL51 protein is a component of HSV-1 virions. To define the location of the UL51 protein further in HSV virions, purified virions were treated with the non-ionic detergent NP40, and then separated into envelope and capsid/tegument fractions. Western blot analysis showed that the majority of UL51 protein was associated with the capsid/tegument fraction, while glycoprotein C (gC) was detected exclusively in the envelope fraction (Fig. 3C).

To determine the subcellular localization of UL51 protein when expressed alone, we constructed the expression plasmid pcDNA-UL51, which expressed the HSV-1 UL51 gene under the control of the human cytomegalovirus (HCMV) immediate-early promoter. Cleavage of pET28-UL51 with NolI and EcoRI released the UL51 open reading frame, which was then ligated into the multi-cloning site of pcDNA3 (Invitrogen) to give pcDNA3-UL51. COS-1 cells were transfected with pcDNA3-UL51 and examined by immunofluorescence staining using the anti-UL51 antiserum. Approximately 15% of cells exhibited strong fluorescence by 48 h post-transfection. Immunofluorescence microscopy showed that the UL51 protein was widely distributed throughout the cytoplasm, although the intensity of fluorescence was higher in the perinuclear region than in the cytoplasm (Fig. 2E, F). This distribution was similar to that observed in infected cells 9 h p.i. There was no difference between the distribution patterns of UL51 protein in cells 24 and 48 h post-transfection.

In the present study, we have raised a rabbit polyclonal antiserum against an HSV-1 UL51 fusion protein and investigated the UL51 gene product in infected cells by using the specific antiserum. The product was identified as 27, 29 and 30 kDa phosphoproteins that were expressed at the late stage of infection and were localized in the cytoplasm of infected cells. Analysis of extracellular virions revealed that the UL51 protein was a component of the virion.

Homologues of the HSV-1 UL51 gene product are conserved among all subfamilies of herpesviruses. These homologues, except that of HCMV, have similar molecular sizes ranging from 200 to 259 amino acids and contain some highly conserved motifs. The UL51 homologues also contain a proline-rich domain in the C-terminal region; for example, about one-quarter of the C-terminal 60 amino acids of HSV-1 UL51 are prolines. These observations might suggest a common role for these UL51 homologues in the herpesvirus replication cycle. Lenk et al. (1997) recently identified the PRV UL51 gene product and demonstrated that it is a 30 kDa virion polypeptide that is present mainly in the nuclear fraction. In contrast to HSV-1, a single 30 kDa UL51 gene product is
detectable in PRV-infected cells and in virions and there is no evidence of post-translational modification. Furthermore, the UL51 protein of PRV has been shown to be expressed with early kinetics, first detected at 3 h p.i. It would be interesting to determine whether these differences from HSV-1 are physiologically significant.

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