Purification and properties of the herpes simplex virus type 1 UL8 protein

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Introduction

The genome of herpes simplex virus type 1 (HSV-1) is a linear dsDNA molecule which contains at least 72 different genes (McGeoch et al., 1988) and three origins of virus DNA replication (Spaete & Frenkel, 1982; Mocarski & Roizman, 1983; Weller et al., 1985). The products of seven genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) are necessary and sufficient for viral origin-dependent DNA synthesis in a transient assay in transfected tissue culture cells (Wu et al., 1988) and all are essential for virus replication and DNA synthesis in tissue culture (Weller, 1991).

Genes UL30 and UL42 encode the catalytic and accessory subunits of the viral DNA polymerase (Dorsky et al., 1987; Wu et al., 1988, Marcy et al., 1990). The UL42 protein interacts with polymerase to increase its rate and processivity (Gallo et al., 1989; Hernandez & Lehman, 1990; Gottlieb et al., 1990). Gene UL29 encodes a ssDNA-binding protein (Weller et al., 1983; Quinn & McGeoch, 1985) and an origin-binding protein is encoded by gene UL9 (Olivo et al., 1988; Weit et al., 1989).

Of the products of the seven replication genes the UL8 protein is the least well characterized. Together with the UL5 and UL52 protein it forms the viral helicase–primase complex in HSV-1-infected cells (Crute et al., 1989). However, a subassembly consisting of only the UL5 and UL52 subunits exhibits all the known enzymatic activities of the three-component complex (Calder & Stow, 1990; Dodson & Lehman, 1991). The presence of UL8 within the helicase–primase complex acts to increase the efficiency of primer utilization by stabilizing the association between nascent oligoribonucleotide primers and template DNA (Sherman et al., 1992).

Immuno-fluorescence experiments suggest that UL8 protein may be important for efficient nuclear uptake of the helicase–primase complex (Calder et al., 1992).

To investigate the UL8 protein further we have developed a rapid and simple two-step purification procedure using insect cells infected with a recombinant baculovirus. This procedure combines one of the four chromatographic steps previously employed by Dodson & Lehman (1991) with a further fractionation using phenyl–Sepharose. We report on the immunological reactivity of the purified UL8 protein, its size in solution and nucleic acid binding properties.

Methods

Cells and recombinant baculovirus. Spodoptera frugiperda (Sf) cells (strain IPLB-SF-21; Kitts et al., 1990) were maintained in TC100 medium (Life Technologies) containing 5% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). The Autographa californica nuclear polyhedrosis virus (ACNPV) recombinant AcUL8 containing the UL8 gene has been described previously (Calder & Stow, 1990). Preparation and titration of virus stocks were carried out as described by Brown & Faulkner (1977) and Matsuura et al. (1987).

Antisera to the UL8 protein. Antisera specific for the amino and carboxy termini of the UL8 protein were prepared in rabbits against amino acids 2 to 16 (DTADIVWVEESVSAI) and 733 to 747 (KFVYFPFDKMSFLFA), respectively, of the 747 residue protein. These oligopeptides were made as branched (octameric) peptides (Possnet et al., 1988; Tam, 1988) by continuous flow Fmoc chemistry as previously described (McLean et al., 1991). The compositions of the branched peptides were determined by amino acid analysis (Cambridge Research Biochemicals) and were in agreement with expectations. Antisera were raised in Sandy Half-Lop rabbits as described (McLean et al., 1991). The amino terminus and carboxy terminus-specific antisera are designated 064 (28.1.91) and 008 (14.9.89) respectively.

Preparation and purification of the UL8 protein. Sf cells (eight 175 cm² flasks each containing approximately 3 × 10⁷ cells) were infected with recombinant baculovirus AcUL8 at an m.o.i of approxi-
mately 5 p.f.u./cell and incubated at 28 °C for 60 to 70 h. The cells were harvested by shaking them into the medium and were centrifuged at approximately 2000 g for 8 min. The pellet was washed by resuspension in 10 ml TBS (20 mM-Tris–HCl pH 7.5, 500 mM-NaCl) per flask and the cells were repelleted. The cell pellet was resuspended in 400 μl/flash of ice-cold buffer (20 mM-triethanolamine–HCl pH 7.5, 10% glycerol, 10 mM-KCl, 1.5 mM-MgCl₂, 1 mM-DTT, 0.5 mM-PMSE, 10 μg/ml leupeptin, 10 μg/ml pepstatin A) and NP40 added to a final concentration of 0.5%. The cell suspension was incubated for 10 min on ice, centrifuged for 2 min at 10000 g and the supernatant was recentrifuged at 17500 g for 8 min at 4 °C. The supernatant from this centrifugation (the crude extract) was frozen at −70 °C until required.

The crude extract was further purified as follows. Thawed extract (3.2 ml) was clarified by centrifugation at 10000 g and the supernatant loaded onto a 2 ml DEAE-Sepharose column (Pharmacia, CL-6B) previously equilibrated in buffer A (20 mM-triethanolamine–HCl pH 7.5, 10% glycerol and 100 mM-NaCl). The column was washed with 10 ml of the same buffer and a 1.4 ml linear gradient of 100 mM- to 350 mM-NaCl in buffer B (20 mM-Tris–HCl pH 8.0, 10% glycerol) was applied. A new column was packed for each purification. The fractions containing UL8, identified by Western blotting using the UL8-specific antisera and corresponding to the leading part of a distinct absorbance peak, were loaded onto a 5 ml phenyl-Sepharose column (Pharmacia, Low substitution Fast flow) equilibrated in buffer C (buffer B containing 100 mM-NaCl). The column was washed with 10 ml of buffer C and protein was eluted with a linear 10 ml gradient ranging from 100% buffer C to 100% buffer D (90% water plus 10% glycerol) at 4 °C. Fractions were analysed as before and those containing the purified UL8 protein were stored at −70 °C.

Measurement of protein concentrations. Protein concentrations were measured using a Pierce bicinchoninic acid protein assay kit with a solution of purified BSA as standard.

SDS–PAGE and Western blotting. The discontinuous buffer system of Laemmli (1970) was used for electrophoresis of the proteins through 10% gels. Separated proteins were either stained with Coomassie blue or blotted onto nitrocellulose membranes as described by Towbin et al. (1979). Transferred proteins were incubated overnight at room temperature with rabbit antiseras used at a 1:200 dilution. Bound antibodies were revealed using horseradish peroxidase–Protein A conjugate and a chromogenic substrate (4-chloro-1-naphthol, Bio-Rad).

Size exclusion chromatography. Purified UL8 protein was concentrated in a Centricon 30 microconcentrator (Amicon) and chromatographed on a 25 ml Superose 12 gel filtration column (Pharmacia) equilibrated in buffer C containing 0.2 mM-NaCl and 0.02% n-octyl β-D-glucopyranoside and run at 0.1 ml/min in the same buffer. Later it was found that the detergent could be omitted without affecting the resolution. Proteins used as size markers were β-amylase (M₅ = 200000), alcohol dehydrogenase (M₅ = 150000), BSA (M₅ = 66000) and carbonic anhydrase (M₅ = 29000) which were obtained from Sigma.

DNA mobility shift assays. The ability of UL8 protein to bind to DNA was assessed with duplex, partial-duplex and single-stranded molecules. A duplex oligonucleotide with the sequence

5' GATCCGCGAAGCGTT CGC ACGTC GTG CCC A
GGCGTT CGC ACGCGT GAA GCAGGG TCTAG 5'

which corresponds to protein binding site I of ori (Weir & Stow, 1990) was used because it has been found to bind several different proteins efficiently in DNA mobility shift assays. It was purified, 32P-5' end-labelled and blunt-ended as described (Weir et al., 1989) to give the dsDNA probe. Alternatively the synthetic duplex was 32P-5' end-labelled with [32P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982) to give the partial-duplex probe. The DNA/RNA hybrid was 5' end-labelled by the same procedure. For the ssDNA probe the oligonucleotide 5' GATCCGCGAAGCGTT CGC ACGTC GTG CCC A was 5' end-labelled using T4 polynucleotide kinase. All labelled oligonucleotides were extracted sequentially with phenol/chloroform, precipitated with ethanol and resuspended in water.

For the mobility shift assay, various amounts of purified UL8 protein (diluted in buffer C containing 0.02% octyl glycoside and 100 μg/ml BSA) were incubated at 37 °C with the radiolabelled probes, 2 μg of ssDNA or dsDNA or 8 ng of partial-duplex DNA in a reaction volume of 20 μl containing 50 mM-HEPES pH 7.5, 10% glycerol, 0.1 mM-EDTA, 0.5 mM-DTT and 2 μg BSA. Where indicated, some reactions contained in addition 50 mM-NaCl and 5 mM-MgCl₂. Purified proteins used as positive controls for binding were HSV-1 UL42 (Gottlieb et al., 1990) and T4 gene 32 product (Boehringer Mannheim). After 20 min 5 μl of loading buffer [25% glycerol, 10 mM-DTT, 0.01% bromophenol blue in TBE (90 mM-Tris base, 90 mM-boric acid, 1 mM-EDTA)] was added and the samples were analysed by electrophoresis in 8% polyacrylamide gels (40:1 acrylamide × 2 N,N'-methylene-bisacrylamide) containing TBE. Gels were run at 120 V for 3 h, dried and the radioactivity was revealed using autoradiography.

Results

UL8-specific antisera

Because no in vitro function has been demonstrated for the UL8 protein, antiseras specific for the amino and carboxy termini of the protein were prepared and used to

![Fig. 1. Reactivity of antiseras made against peptides from the amino and carboxy termini of the UL8 protein with extracts of cells infected with recombinant virus AcUL8 (lanes 5 to 8) or the parental wild-type virus, AcNPV (lanes 1 to 4). The proteins were separated on a 10% SDS-polyacrylamide gel and after transfer to nitrocellulose were reacted with the antiseras. The reactivities of both the immune (odd-numbered lanes) and pre-immune (even-numbered lanes) sera from rabbits immunized with either the amino-terminal (lanes 1, 2 and 5, 6) or carboxy-terminal (lanes 3, 4 and 7, 8) peptides are shown.](image-url)
HSV-1 UL8 protein

Fig. 2. Column chromatography of the UL8 protein. Extracts of Sf cells infected with recombinant baculovirus AcUL8 were fractionated first in DEAE-Sepharose (a) then in phenyl-Sepharose (b) as described in Methods. The A_{280} was monitored and the presence of the UL8 protein determined by Western blotting. UL8 protein eluted in fractions between the arrowheads.

monitor purification of the protein and to provide evidence for its structural integrity. The reactivities of these antisera are shown in Fig. 1. Both immune sera reacted in Western blots with extracts of cells infected with recombinant baculovirus AcUL8 but not the parental wild-type virus. As expected, the pre-immune sera did not react with either extract.

Purification of the UL8 protein

Fig. 2 shows the chromatographic separation of the UL8 protein in the two steps of the purification scheme, DEAE-Sepharose (a) and phenyl-Sepharose (b). Fractions containing the protein were identified by Western blotting using antiserum specific for its amino or carboxy terminus and are indicated between the arrowheads on the figure. The UL8 protein eluted between 240 mM- and 340 mM-NaCl from the DEAE-Sepharose column and as a symmetrical peak of A_{280} at the end of the gradient (no salt) from the phenyl-Sepharose column.

The proteins present in the crude extract (CE) and at two stages of purification are shown in Fig. 3. The UL8 protein bound efficiently to both the DEAE-Sepharose and phenyl-Sepharose columns as judged by its absence from the flowthrough (FT). Densitometric scanning of the gel indicated that the protein was approximately 95% homogeneous after the second column elution.

The purified protein was reactive with both amino and carboxy terminus-specific antisera (Fig. 4) indicating that both termini were intact and that proteolytic degradation had not occurred during purification. A yield of approximately 1.5 mg of purified protein was obtained from 2.4 x 10^8 cells (equivalent to eight 175 cm² flasks).

The UL8 protein is monomeric in solution

To determine whether purified UL8 protein exists as a monomer or multimer in solution it was subjected to gel filtration chromatography as described in Methods. The...
Fig. 4. Reactivity of the purified UL8 protein with both amino-terminal and carboxy-terminal peptide antisera. Samples of UL8 protein (2 μg) were subjected to acrylamide gel electrophoresis and Western blotting and reacted with antibodies raised against peptides from the amino (lane 1) or carboxy (lane 2) terminus.

Fig. 5. Size exclusion chromatography of the purified UL8 protein. The curve shows the A₂₈₀ of the purified protein (peak absorbance 0.2). Protein markers were β-amylase, alcohol dehydrogenase, BSA and carbonic anhydrase (Mr’s are 200000, 150000, 66000 and 29000, respectively). These eluted sequentially from the column and the positions of the peak fractions are indicated with circles.

protein eluted as a symmetrical peak (Fig. 5) with an estimated Mr of 75000, a figure which is in good agreement with its predicted Mr of 79921 (McGeoch et al., 1988). The error in this measurement is ±5000, calculated from a maximum error of ±0.1 ml in elution volume. This observation indicates that the protein exists as a monomer in solution.

Gel mobility shift assays do not detect binding of the UL8 protein to nucleic acids

A gel mobility shift assay was used to investigate whether purified UL8 protein bound to nucleic acids. Fig. 6 shows the results after incubating protein samples with either 2 ng of dsDNA probe in buffer with no salt (lanes 1 to 6) or 2 ng of ssDNA probe in buffer with 50 mM-NaCl (lanes 7 to 11). UL42 protein which is known to bind to dsDNA (Bayliss et al., 1975; Gallo et al., 1988) was used as a positive control and caused a distinct mobility shift of the probe (arrow, lane 1). In contrast, up to 60 ng of purified UL8 protein caused no shift in mobility (lanes 2, 3 and 4) of the double-stranded probe. Further experiments in which up to 600 ng of UL8 protein was used showed no mobility shift, nor was any retardation seen when 50 mM-NaCl was included in the incubation buffer (data not shown). Reactions were also performed at 0 °C, 25 °C and 37 °C and again no retarded complex was observed. As expected, T4 phage gene 32 protein, which binds cooperatively to ssDNA (Alberts et al., 1969; Alberts & Frey, 1970) caused a major retardation of the ssDNA probe so that it migrated just below the top of the gel (lane 7). There was no evidence of any retardation of the probe with up to 600 ng of UL8 protein (lanes 8 and 9). Incubation of the probe and UL8 protein in the absence of salt also failed to demonstrate any binding (data not shown). In other experiments UL8 protein did not bind to partial-duplex DNA to which UL42 bound nor to an RNA/DNA hybrid [poly(rA)–p(dT)₁₂₋₁₈, Pharmacia] to which T7 RNA polymerase bound (data not shown). These experiments suggest that UL8 protein does not bind to nucleic acids.

Discussion

The purification scheme which we have described is simple and rapid. A yield of 1.5 mg of about 95% pure UL8 protein can be achieved in 4 h from 2.4 x 10⁶ Sf cells infected with recombinant baculovirus. The usefulness of the DEAE–Sephrose chromatography step in the purification of UL8 protein was previously reported as part of a four-stage purification scheme (Dodson & Lehman, 1991). It is not possible to compare the degree of purification afforded by the various steps of the two schemes since SDS–polyacrylamide gels of the proteins present after each step in the scheme of Dodson & Lehman (1991) were not presented. The UL8 protein has also been partially purified by DEAE–Sephacel chromatography from Sf9 cells triply infected with baculovirus recombinants to produce the UL5/UL8/UL52 complex (Sherman et al., 1992). These authors estimated that the protein was approximately 30% pure.
The UL8 protein, purified as described here, is largely free from proteolytic degradation products as judged by its reactivity with both the amino and carboxy terminus-specific antisera. The possibility that a few amino acids (fewer than 10) have been lost from either end cannot be rigorously excluded because the antisera were raised against peptides 15 amino acids long and some epitopes might remain. This possibility, however, seems unlikely.

Using the purified UL8 preparation we have shown that the protein exists as a monomer in solution and does not detectably bind to nucleic acid under conditions where binding of other proteins can be readily demonstrated. Such behaviour has been found previously with a protein involved in DNA replication in the proliferating cell nuclear antigen, an auxiliary protein for DNA polymerase that enhances its processivity (reviewed by Wang, 1991), but which does not bind to DNA in the absence of replication factor C (Tsurimoto & Stillman, 1991). The possibility that the UL8 protein did not bind because it had been denatured is unlikely because it remained soluble at all stages of the purification procedure. However, the possibility remains that a different type of assay might demonstrate binding of the protein to a nucleic acid.

In HSV-1-infected cells the UL8 protein occurs as part of the helicase–primase complex together with the UL5 and UL52 proteins (Crute et al., 1989) although its presence does not appear to affect the in vitro enzymatic properties of this complex (Calder & Stow, 1990; Dodson & Lehman, 1991). It will be interesting to determine whether the purified UL8 protein can interact with the UL5 plus UL52 subassembly in vitro or affect its subcellular localization in vivo and whether it can bind to nucleic acids in the presence of UL5 and UL52.

References


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