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The effect of the UL42 protein on the DNA polymerase activity of the catalytic subunit of the DNA polymerase encoded by herpes simplex virus type 1

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The effect that the UL42 protein of herpes simplex virus type 1 has on the DNA polymerase activity of the DNA polymerase catalytic subunit (Pol) of the same virus has been investigated. The observed effects are critically dependent on the salt used and its concentration, such that the UL42 protein may inhibit, have little or no effect on, or activate the Pol activity, depending on the conditions used. The observed effects are due to the values for $K_{m_{app}}$ for activated DNA and $V_{max_{app}}$ for Pol and the Pol-UL42 protein complex differently varying with salt concentration.

DNA polymerase; UL42 protein; Herpes simplex virus

1. INTRODUCTION

Origin-dependent replication of the herpes simplex virus type 1 (HSV-1) genome requires seven viral genes [1]. Biochemical functions have been identified for the products of these genes and this work has been reviewed [2,3]. Briefly, UL9 encodes a sequence-specific origin-binding protein; UL5, UL8, and UL52 encode the 3 proteins that form a DNA helicase/DNA primase complex; UL29 encodes a single-stranded DNA-binding protein; UL30 and UL42 encode, respectively, the catalytic and accessory subunits of the heterodimeric DNA polymerase. The UL42 product has also been identified as a double-stranded DNA-binding protein. In addition to the above viral gene products, it is possible that one or more so-far unidentified host cell proteins may also play a role but, if this is so, then infected insect cells are as able to provide the necessary factor(s) as are mammalian cells [4].

When purified from HSV-1-infected cells the viral DNA polymerase is obtained as a 1:1 complex [5] of the 136 kDa catalytic subunit (Pol) (which has structural similarities to DNA polymerases alpha [6] and delta [7]) and the 65 kDa UL42 protein.

The inter-relationship between the Pol subunit and the UL42 protein is unclear. It was suggested [8] that the two subunits may be separated by centrifugation through a glycerol gradient, a step in which much of the polymerase activity was apparently lost. When the UL42 protein was added back to the Pol, the polymerase activity was stimulated by up to 10 times. However, the Pol and UL42 protein subunits were not separable by sucrose gradient centrifugation [5], and other workers ([9], G.J. Hart, unpublished work) have been unable to dissociate the Pol–UL42 protein complex except under conditions that apparently caused total loss of polymerase activity.

The recent use of the baculovirus, *Autographa californica* nuclear polyhedrosis virus has facilitated the study of many of the HSV-1 replication proteins, and is particularly useful for studies of the Pol and UL42 proteins. By using appropriate recombinant baculoviruses both subunits of the HSV-1 DNA polymerase have been obtained free of the other subunit [9–11].

It has been reported [10,11] that the specific activity of the purified Pol is very similar to that of the DNA polymerase (Pol–UL42 protein heterodimer) isolated from HSV-1-infected mammalian cells. The conclusion drawn from this was that any stimulatory activity by the UL42 protein on the Pol activity was slight, in contrast to the results of Gallo and co-workers [8], although the UL42 protein was found to increase the processivity of the polymerisation ([10], A. Owsianka and H.S. Marsden, Institute of Virology, Glasgow, personal communication). Other workers [9] report that the UL42 protein increases both the processivity and the activity of the Pol, but only if the UL29 gene product (also known as ICP8) is present.

As the exact role that the UL42 protein has in the function of the DNA polymerase is rather uncertain, we

Abbreviations: HSV-1, Herpes simplex virus type 1; Pol, catalytic subunit of DNA polymerase of HSV-1; PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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decided to investigate in some detail the interaction between the two proteins. In this report we have examined the effect that the UL42 protein has on the activity of Pol. The observed effects are critically dependent on the salt concentration, such that the UL42 protein may inhibit, have little or no effect on, or activate the Pol activity, depending on the conditions used. Further, it is shown that the K_m for activated calf thymus DNA for the Pol-UL42 protein complex is little affected by the concentration of salt, a result in marked contrast to that obtained with Pol alone, where increasing salt concentration leads to a large increase in the value for K_m for activated DNA.

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

Recombinant baculovirus, BP58, containing the HSV-1 UL30 gene [11], and AcUL42, containing the UL42 gene [4], were obtained from Dr. D.M. Coen, Harvard Medical School, MA, USA, and Dr. N.D. Stow, Institute of Virology, Glasgow, UK, respectively.

Spodoptera frugiperda (Sf9) cells were cultured and baculoviruses were propagated as previously described [12].

200 ml spinner cultures of Sf9 cells (10^6 cells/ml) were infected with the appropriate recombinant baculovirus (BP58 for Pol, or AcUL42 for UL42) at a multiplicity of infection of approximately 10 PFU/cell. The cells were incubated at 28°C for a further 48–54 h, and then collected by centrifugation. They were washed in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl) and stored frozen at −20°C until required.

2.2. *Protein purification*

The purification procedures used for the Pol and UL42 proteins were based on previously published methods [10,11]. The methods will be outlined with only the major modifications described in detail. Cells (from one 200 ml spinner culture) were allowed to thaw and were suspended in 5 ml of 10 mM sodium phosphate buffer, pH 7.5, containing 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, and 10 μg/ml of each of pepstatin A and aprotinin. The cells were lysed, the nuclei were collected by centrifugation, and resuspended in 2.5 ml of the above buffer to which was added an equal volume of 3.4 M NaCl dissolved in the same buffer. After 30 min the nuclear suspension was homogenised, and nuclear membranes were removed by centrifugation. As the subcellular localisation of the proteins in the expression system used was unknown, the two supernatant fractions were combined and further clarified by centrifugation (40,000 × g/1 h). The resultant supernatant fraction was dialysed overnight against 2 × 250 ml of 20 mM HEPES/NaOH buffer, pH 7.5, containing all the additions used in the homogenisation buffer plus 50 mM NaCl, and 10% (v/v) glycerol (buffer A). Fresh PMSF (1 mg in 50 μl of ethanol) was added, and the small amount of protein that had precipitated during the dialysis was removed by centrifugation. The supernatant was applied to a column (1.6 × 5 cm) of P11 cellulose phosphate (Whatman). After the column had been washed with about 20 ml of buffer A, proteins were eluted with a 240 ml linear gradient from 0.05 to 0.85 M (Pol) or 0.75 M (UL42 protein) NaCl in buffer A. The flow rate was 20 ml/h and fractions of about 4.5 ml were collected. Those fractions containing DNA polymerase activity due to HSV Pol, or the major peak of UL42 protein, were combined and concentrated to about 4 ml by ultrafiltration, which was then dialysed against 2 × 300 ml of buffer A.

Pol was further purified by chromatography on a column (1.3 × 4.0 cm) of single-stranded DNA agarose (Bethesda Research Laboratories). Proteins were eluted from the column with a linear gradient from 0.05 to 0.65 M NaCl in buffer A at a flow rate of 12 ml/h. Fractions of 3 ml were collected. Those that contained the highest amounts DNA polymerase activity were combined and stored at −70°C until used. Attempts to purify the Pol further resulted in large losses of DNA polymerase activity.

UL42 protein was further purified on a column (1.3 × 3.5 cm) of double-stranded DNA cellulose (Sigma). Proteins were eluted from the column with a 120 ml linear gradient from 0.05 to 0.65 M NaCl in buffer A at a flow rate of 12 ml/h. Fractions of about 3 ml were collected. Those that contained the UL42 protein were combined and stored at −70°C until used. Immediately before use samples of UL42 protein were dialysed overnight against buffer A.

2.3. *Enzyme and protein assays*

Standard DNA polymerase assays were made at 37°C in 75 mM Tris-HCl buffer, pH 7.5, containing 6.5 mM $MgCl_2$, 83 μM dATP, 83 μM dCTP, 83 μM dGTP, 1.67 mM 2-mercaptoethanol, 0.011 mg/ml activated calf thymus DNA, 0.42 mg/ml bovine serum albumin, and 2.5 μM [^{3}H]dTTP (80–85 Ci/mmol). During the polymerase purification assays contained 50 mM KCl. When the effect of salt concentration on the activities of Pol and the Pol–UL42 protein complex was studied, concentrations of KCl, NaCl, or $(NH_4)_2SO_4$ were varied as appropriate, and as detailed in section 3 of this report. The concentration of NaCl carried over into the assays from the Pol or Pol–UL42 protein complex solutions was 8.5 mM. During detailed kinetic studies the concentration of activated calf thymus DNA was varied over a range that encompassed the concentration equivalent to K_m under the conditions used. 25 μl samples were removed from assays at intervals over the first 20–25 min of reaction and were spotted onto a DEAE-filter mat, previously soaked in 0.1 M Na_4EDTA and air dried. (This pre-treatment of the filter mat had the effect of immediately stopping the polymerase reaction and avoided the problem of high blank counts, which were seen if the filter mat was not so pre-treated.) The filter mat was washed for 3 × 10 min with 5% (w/v) Na_2HPO_4, then for 2 × 5 min with water and finally for 2 × 30 s with industrial methylated spirits. The filter mat was dried in a current of warm air and counted in an LKB (Milton Keynes, Buckinghamshire, UK) beta-plate scintillation counter according to the manufacturer's directions for 1–3 min per sample position.

The presence of UL42 protein was determined by analysis of dot-blots of samples of fractions by using an antibody partially purified from the serum of rabbits that had been challenged with a synthetic peptide (linked to keyhole limpet hemocyanin) corresponding to the C-terminal 10 amino acid residues of the UL42 protein. Rabbit IgG was then detected with an AuroProbe BLplus kit (Janssen Biotech).

3. RESULTS AND DISCUSSION

3.1. *Purification of Pol and UL42 protein*

The behaviour of Pol during chromatography on cellulose phosphate and single-stranded DNA agarose was as described previously [11].

Chromatography on P11 cellulose phosphate separated the UL42 protein into a major peak (approximately 75% of the total UL42 protein) eluted by 0.2–0.25 M NaCl, and a minor peak (about 25% of the total) eluted by 0.3–0.35 M NaCl. The UL42 protein from the two peaks co-ran on SDS-polyacrylamide gel electrophoresis, with an apparent M_r of 65,000, and both were able to stimulate the activity of Pol at high concentrations of salt (see below). The differences between UL42 protein from the two peaks are not yet apparent, and only the major peak has been investigated in detail. Upon storage at −70°C the final product slowly lost, over about 3 months, its ability to stimulate the activity of Pol.

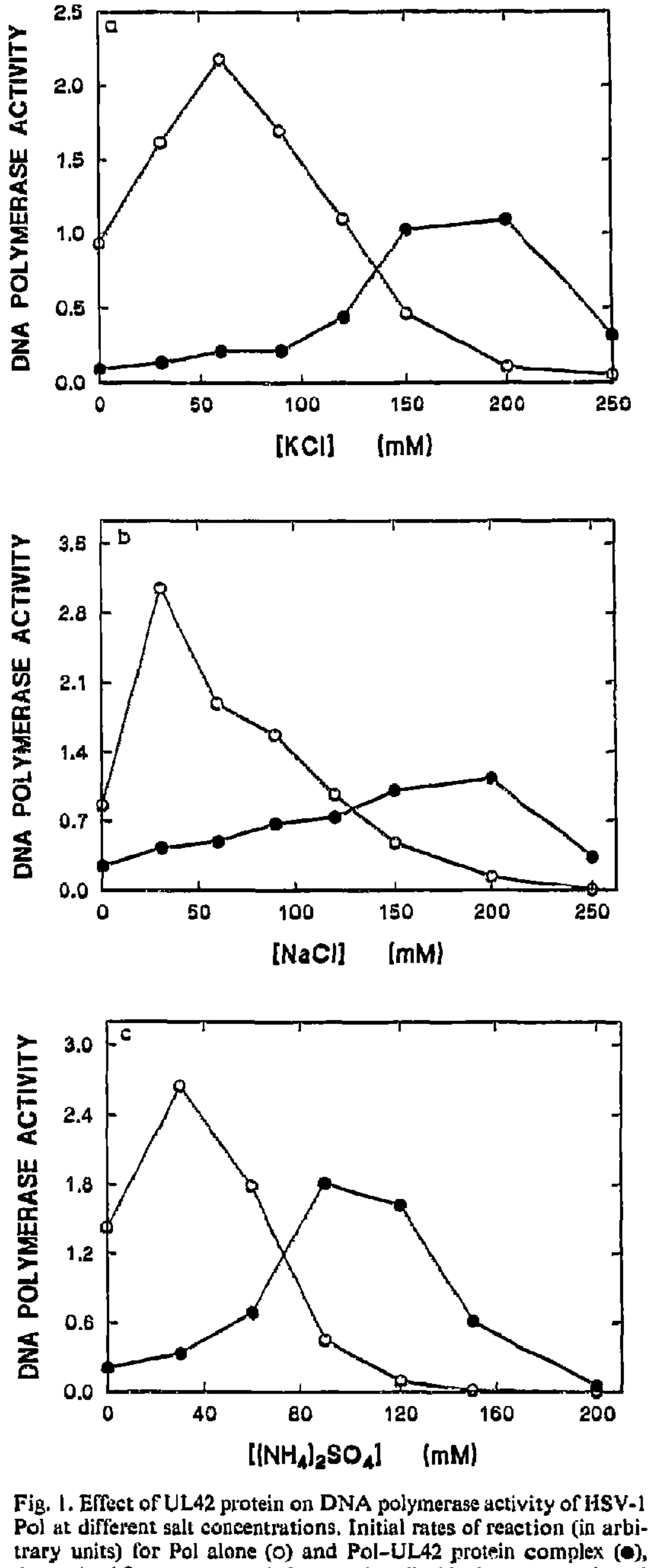

Fig. 1. Effect of UL42 protein on DNA polymerase activity of HSV-1 Pol at different salt concentrations. Initial rates of reaction (in arbitrary units) for Pol alone (○) and Pol-UL42 protein complex (●), determined from assays carried out as described in the text, are plotted against the concentration of (a) KCl, (b) NaCl, (c) $(NH_4)_2SO_4$ present in the assays. 8.5 mM NaCl was carried over into assays from the protein solutions.

3.2. *Effect of salt concentration on DNA polymerase activity of Pol and Pol-UL42 protein complex*

The effects that different concentrations of KCl, NaCl and $(NH_4)_2SO_4$ have on the DNA polymerase activities of HSV-1 Pol, and the same amount of Pol plus an equivalent amount (that amount producing the maximum effect on the DNA polymerase activity of Pol after 10 min of pre-incubation on ice) of the UL42 protein using activated calf thymus DNA as template/primer are shown in Fig. 1.

These results, which have been reproduced with several different preparations of Pol and UL42 protein, show that the measured DNA polymerase activity is critically dependent both on the nature and on the concentration of the salt present in the assays. Also, Pol and the Pol–UL42 protein complex have very different activity against salt concentration profiles. This means that the effect that the UL42 protein has on the activity of the Pol is dependent on the salt concentration at which the activity is measured, and it is only at high salt concentrations (e.g. >150 mM KCl) that the UL42 protein exerts a stimulatory effect on the DNA polymerase activity of Pol. At low concentrations of salt the UL42 protein has an inhibitory effect on the DNA polymerase activity of the Pol, while there is an intermediate range of salt concentrations over which the UL42 protein has little or no effect on the polymerase activity of Pol.

The apparent discrepancies in the literature regarding the effect that the UL42 protein has on the DNA polymerase activity of Pol now seem to be explicable. The reported activation [8] was observed under high salt conditions (100 mM Tris buffer, pH 8.0, 200 mM KCl), while lack of any effect [10,11] was found under conditions (20 mM Tris-HCl, pH 7.5, 150 mM $(NH_4)_2SO_4$) that seem similar to those (75 mM Tris-HCl, pH 7.5, 70 mM $(NH_4)_2SO_4$; Fig. 1) under which the UL42 protein has little or no effect on the polymerase activity of Pol.

3.3. *Effect of KCl concentration on* $K_{m_{app}}$ *for activated DNA, and* $V_{max_{app}}$ *for Pol and Pol–UL42 protein complex*

Values for $K_{m_{app}}$ for activated calf thymus DNA and $V_{max_{app}}$ were determined from plots of [DNA]/initial rate against [DNA] (not shown) both for Pol alone and for Pol–UL42 protein complex at several concentrations of KCl. Fig. 2 shows how the values for $K_{m_{app}}$ for activated calf thymus DNA and $V_{max_{app}}$ vary with concentration of KCl.

The results in Fig. 2 provide an explanation for many of the observations presented in Fig. 1. Considering, first, high salt concentrations, e.g. 200 mM KCl, $V_{max_{app}}$ for Pol–UL42 protein complex is only about 30% greater than that for Pol alone. However, the value for $K_{m_{app}}$ for activated DNA for Pol is >20 times that found for Pol–UL42. This means that with the standard assay amount (11 µg/ml) of activated DNA, Pol is working at only about 20% of $V_{max_{app}}$, whereas Pol–UL42 protein

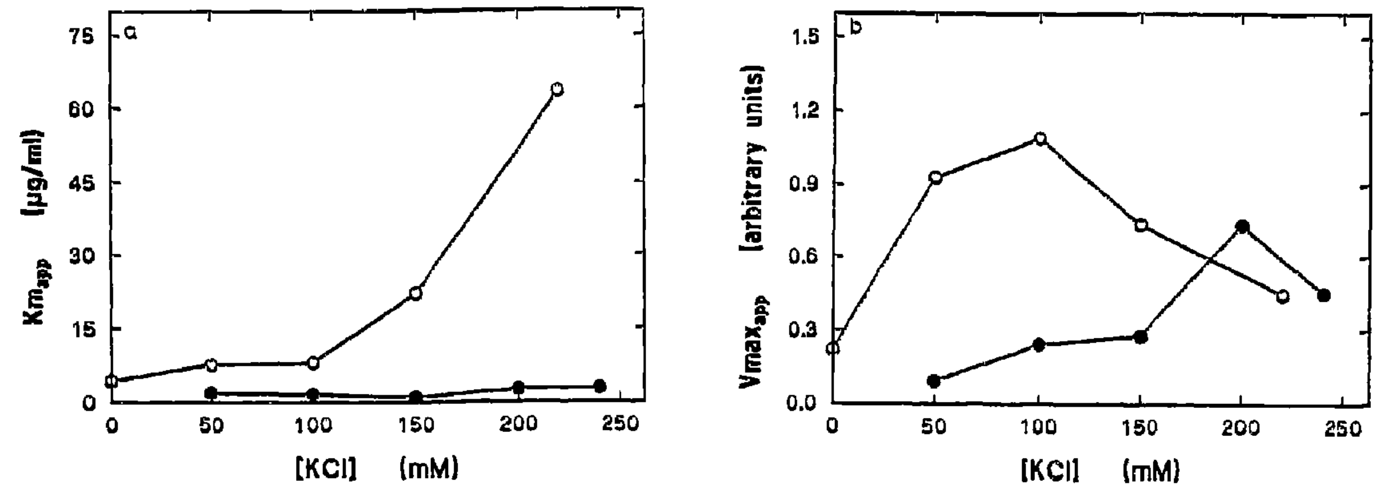

Fig. 2. Effect of KCl concentration on $K_{m_{app}}$ for activated DNA and $V_{max_{app}}$ of HSV-1 Pol and Pol–UL42 protein complex. Values for (a) $K_{m_{app}}$ for activated DNA and (b) $V_{max_{app}}$, determined as described in the text, are plotted against the concentration of KCl present in the assays. Results are shown for Pol alone (○) and Pol–UL42 protein complex (●).

complex is operating at about 80% of its $V_{max_{app}}$. So, under standard assay conditions but with 200 mM KCl, the observed rate with Pol–UL42 protein complex should be about 5.5 times the rate with Pol alone. The ratio of these rates from Fig. 1 is about 7.

At low concentrations of salt (e.g. <50 mM KCl) values for $K_{m_{app}}$ for Pol and Pol–UL42 protein complex differ only by a factor of about 3, and observed differences in rate under the standard assay conditions are mostly due to the large (>12-fold) differences in $V_{max_{app}}$. It is not at present clear why the Pol–UL42 protein complex should be so much less active at low concentrations of salt than Pol alone. This may reflect changes in the conformation of Pol when it binds to the UL42 protein, although the differences in values for $K_{m_{app}}$ for activated DNA at low salt concentrations are much less than the differences in values for $V_{max_{app}}$. Also, values for $K_{m_{app}}$ for dTTP (0.1–0.25 μM; G.J. Hart, unpublished work) are almost identical for both Pol and Pol–UL42 protein complex.

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