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A HELIX-DESTABILISING PROTEIN FROM HERPES SIMPLEX VIRUS TYPE I INFECTED CELLS WHICH SPECIFICALLY STIMULATES THE VIRUS INDUCED DNA POLYMERASE ACTIVITY IN VITRO

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SUMMARY: We have isolated a protein fraction from HSV-1 infected cells which binds specifically to single-stranded DNA, facilitates a lowering of the melting temperature of poly[d(A-T)] and specifically stimulates the activity of the homologous virus-induced DNA-dependent DNA polymerase in vitro. These are major characteristics of a helix-destabilising protein, exemplified by the prokaryotic gene 32 protein.

A number of non-enzymic prokaryotic proteins have previously been described which have the general properties in common described in the summary above. These proteins have been referred to as DNA melting proteins, DNA extending proteins, DNA unwinding proteins and more recently as helix-destabilising proteins. The prokaryotic gene 32 protein encoded in the genome of bacteriophage T4 (1) is the most well-characterised of this type and properties of other helix-destabilising proteins are often compared with it.

A variety of proteins with some properties in common have also been reported from eukaryotic sources, summarised by Falaschi et al (2). Several of these proteins exhibit co-operative binding to single-stranded DNA. and will specifically stimulate the homologous DNA polymerase on an appropriate template (3). In some cases these properties are dependent on the state of phosphorylation (4).

The genome of herpes simplex virus is a linear, double-stranded DNA molecule of molecular weight 100 x 10^6 (5). This quantity of DNA has the potential to code for about 100 proteins of mol.wt. 40,000; of these 50 or more induced

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Abbreviations: HDP; helix-destabilising protein

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in virus-infected cells have been detected using single-dimensional gel-electrophoresis (6,7) and many more by 2D-electrophoresis (8). One of these induced proteins is a DNA-dependent DNA polymerase which is encoded in the virus- genome and is distinct from the host cell DNA polymerases (9). In view of the growing number of reports which describe a helix-destabilising protein specifically stimulating only homologous DNA polymerase we hypothesised that such a protein might, therefore, be present in HSV-infected cells. This report describes the results of our search for this putative protein.

MATERIALS AND METHODS

Buffers: Hypotonic buffer was 20mM Tris-HCl, pH 7.5 at 4°C, 0.5mM dithiothreitol; High salt buffer, 20mM Tris-HCl, pH 7.5 at 4°C, 0.5mM dithiothreitol, 3.4M KCl, 10mM EDTA, 1 mg/ml bovine serum albumin; Buffer A, 20mM Tris-HCl, pH 7.5 at 4°C, 50mM KCl, 1mM EDTA, 0.5mM dithiothreitol and 10% (v/v) glycerol; Buffer B was 50mM Tris-HCl, pH 7.5 at 4°C, 0.5mM dithiothreitol, 0.2% (v/v) NP40 and 20% (v/v) glycerol.

Cell culure and growth of herpes simplex virus: Baby hamster kidney cells, BHK-21/C13 (10) were routinely grown in monolayer culture as previously described (11) and infected with HSV-1 strain 17 (Medical Research Council, Institute of Virology, Glasgow, U.K.) at a multiplicity of infection of 20 in Eagle's medium containing 3% (v/v) calf serum (EC3). Proteins were extracted 16h after infection.

Isolation of total cell protein: Cells were removed from monolayers by scraping with a rubber "policeman" after first being washed with phosphate-buffered saline. They were resuspended in hypotonic buffer at a concentration of 1×10^7 to 3×10^7 cells/ml. The proteinase inhibitor, phenyl methyl sulphonyl fluoride, was added at 20µg/ml and the cells then subjected to ultrasonic disruption. An equal volume of high salt buffer was added to the disrupted cells to give a final concentration of 1.7M KCl, 5mM EDTA and 500µg/ml albumin and allowed to stand for 40 min. Insoluble material which formed during this time was removed by centrifuging for 60 min at $35,000g_{av}$ (MSE 18, 8 x 50 ml rotor) and the supernatant dialysed extensively against 3 x 1.5 litres of buffer A. After dialysis the extract was cleared by centrifugation ($18,000g_{av}$ for 30 min using an 8 x 50 ml rotor in the MSE 18 centrifuge). The supernatant was used for subsequent DNA-cellulose chromatography.

This supernatant contained only about 0.2% of the DNA originally present in the initial cell lysate, as measured by the recovery of acid-insoluble radioactivity after previously growing cells in the presence of ^{3}H-thymidine throughout the cell growth and the 16h of virus infection. About 50% of the proteins which were synthesised during the infection period were recovered in the supernatant (recovery of acid-insoluble radioactivity after virus-infection of cells in the presence of a ^{3}H-labelled amino acid mixture).

Single-stranded DNA-cellulose column chromatography: The DNA-cellulose was prepared essentially by the method of Alberts and Herrick (12). A solution of Salmon sperm DNA (at 2mg/ml in 10mM Tris-HCl, pH 7.5 at 20°C, 1mM EDTA) was boiled for 15 min, to denature the DNA, and then rapidly cooled in ice-water. The solution was then adjusted to 20mM Tris-HCl, pH 7.4 and mixed with the cellulose (1g cellulose : 3 ml of the DNA solution). Routinely 50g of DNA-cellulose was made at a time and used immediately or stored for up to 4 months at 4°C in the Tris-buffer.

The DNA-cellulose was degassed by gentle shaking under vacuum before pouring into the columns (2.2 x 20 cm). These were then equilibrated with buffer A and the proteins eluted at 4°C at a flow rate of 20ml/h; 5 ml fractions were collected.

Sephadex G-200 gel-filtration: Sephadex, swollen in buffer B, was degassed and poured into columns of dimension 2.2 x 30cm. These were further washed with buffer B before loading protein samples. Columns were run at 4°C at a flow rate of 2 ml/h and 2ml fractions were collected.

Helix-destabilising assays: The HDP activity was determined by measuring the effect the protein fractions had on the melting temperature (Tm) of poly [d(A-T)] measured at 254 nm. The assays were performed in microcuvettes in a final volume of 500 µl prepared by mixing 250 µl of protein solution with 200 µl of 10mM potassium phosphate, pH 7.8 at 4°C, containing 10%(v/v) glycerol, 50 µl of 100mM $MgCl_2$ and 10 µl of poly [d(A-T)] in Tris-HCl, pH 8.8 at 20°C, 1mM EDTA. The reference sample contained 10 ul of buffer without poly [d(A-T)]. For measurement of the T_m of poly [d (A-T)] in the absence of protein 250 µl of 1mM Tris-HCl, pH 7.4 at 4°C, 0.1mM EDTA was added instead of theprotein solution. Protein samples eluted from DNA-cellulose were desalted by centrifugation through Sephadex G-25(13) before being used in the melting assays.

Purification and assay of the HSV-induced DNA polymerase: The purification procedures used were based on those of Powell and Purifoy (14) and are summarized elsewhere (15). Assays were initiated by adding 50 µl of the enzyme sample to 150 µl of solution consisting of 100 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 3mM $MgCl_2$, 0.26mM each of dATP, dCTP and dGTP, 5µM [^{3}H] dTTP (2Ci/mmol), 20 µg of activated" salmon sperm DNA and 133mM KCl. Samples were incubated for 20 min at 37°C and the reaction stoppedby adding 200 µl of ice-cold 10%(w/v) trichloroacetic acid containing 2% (w/v) sodium pyrophosphate. Acid-insoluble material was subsequently collected on glass-fibre filters for radioactivity determination (16).

RESULTS

Fig 1 shows the elution profiles obtained after chromatography of total cell protein from mock-infected and virus-infected cells on columns of single-stranded DNA-cellulose. The majority of the protein in each case did not bind to the DNA-cellulose, although protein was eluted at each of the salt concentrations used. The ability of each of the fractions to decrease the T_m of poly [d(A-T)] was measured after desalting. Uncharacteristic melting profiles were obtained using the 0.15M KCl and the 0.3M KCl fractions from the mock-infected cells in that the initial absorbance at 260nm was greater than that of the other samples and this then decreased considerably with increasing temperature before finally increasing as the poly[d(A-T)] melted. It was difficult, therefore, to measure accurately the T_m in the presence of these fractions. However, numerous experiments (about 12) demonstrated a reproducibly distinct lowering of the T_m by the 0.3M KCl from the virus-infected cells. Fig. 2 demonstrates that a normal melting

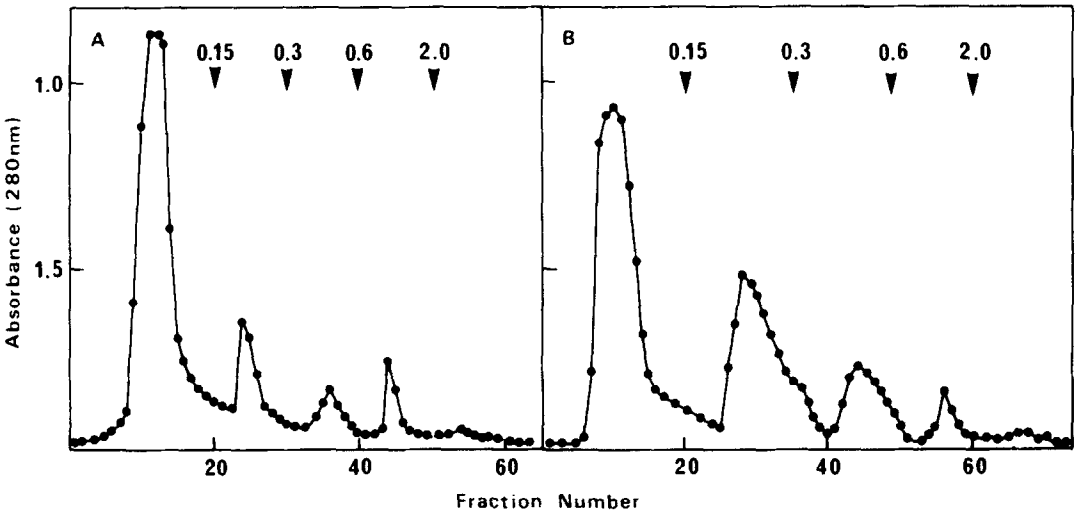

Fig. 1 Single-stranded DNA-cellulose column chromatography. Total cell protein (about 600 mg) from mock-infected cells (A) or virus-infected cells (B) was applied to the columns, which were then eluted by the step-wise addition of KCl solutions at the molarities shown in the figures.

profile was obtained in the presence of this protein fraction. A depression in the T_m of some 15°C was obtained at a protein : poly [d(A-T)] ratio of 30 : 1; by comparison the 0.3M KCl fraction from DNA-cellulose chromatography of proteins from the mock-infected cells did not affect the T_m at a ratio greater than this of 50 : 1.

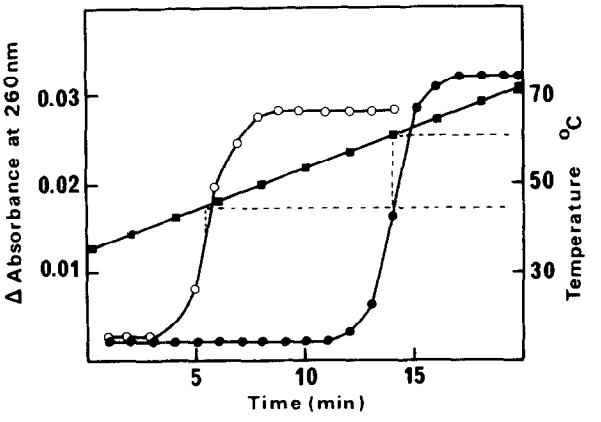

Fig. 2 Depression of the melting temperature of poly [d(A-T)] in the presence of the 0.3M KCl fraction from DNA-cellulose chromatography of total cell protein from HSV-infected cells. (o) without added protein, T_m = 61°C (o) with 0.3M KCl fraction, T_m = 45°C; the final protein concentration in the assay was 188 µg/ml and the protein : poly [d(A-T)] ratio was 30 : 1.

Since a lowering of T_m would be expected if deoxyribonuclease action were to decrease the molecular size of the poly [d(A-T)], and especially since a virus-coded deoxyribonuclease is induced in HSV-infected cells (17), we determined the location of nuclease activity in the DNA-cellulose fractions and then took steps to remove it from the 0.3M KCl fraction. After incubating duplex DNA of bacteriophage λ with each of the DNA-cellulose fractions (using identical protein : DNA ratios) followed by both neutral and alkaline 1% agarose gel-electrophoresis to ascertain the extent of double-strand breaks, single-strand breaks or even extensive exonucleolytic nuclease action, we established that most nuclease activity was present in the flow-through and the 0.15M KCl fractions after chromatography of proteins from both mock-infected and virus-infected cells. Since the 0.3M KCl fraction did contain some, however, this was removed by gel-filtration chromatography on Sephadex G-200 columns. Fig. 3 shows the protein elution profiles and the ability of the different fractions to depress the T_m of poly [d(A-T)]. No depression was observed with proteins from mock-infected cells but was clearly detectable using fractions from the infected cells (fractions 7-19; the protein : poly [d(A-T)] ratio was 2 : 1. The ratio for comparable fractions from the mock-infected cell proteins was 4 : 1). Some deoxyribonuclease activity was detectable in earlier fractions (7-12) but not in later fractions from these columns (similar protein concentrations were used to assay each fraction).

Stimulation of homologous DNA polymerase : Table 1 shows that those fractions from the sephadex chromatography of infected-cell protein (Fig. 3b) which caused a lowering of the T_m of poly [d(A-T)], but were free of nuclease, also contained a component(s) which specifically stimulated the HSV-induced DNA polymerase. This stimulation was up to 4-fold with the 40 µg of protein available. No stimulation of host cell DNA polymerase α or β was detectable.

DISCUSSION

The protein fraction from HSV-1 infected cells that we have described possess major characteristics of a helix-destabilising protein in that

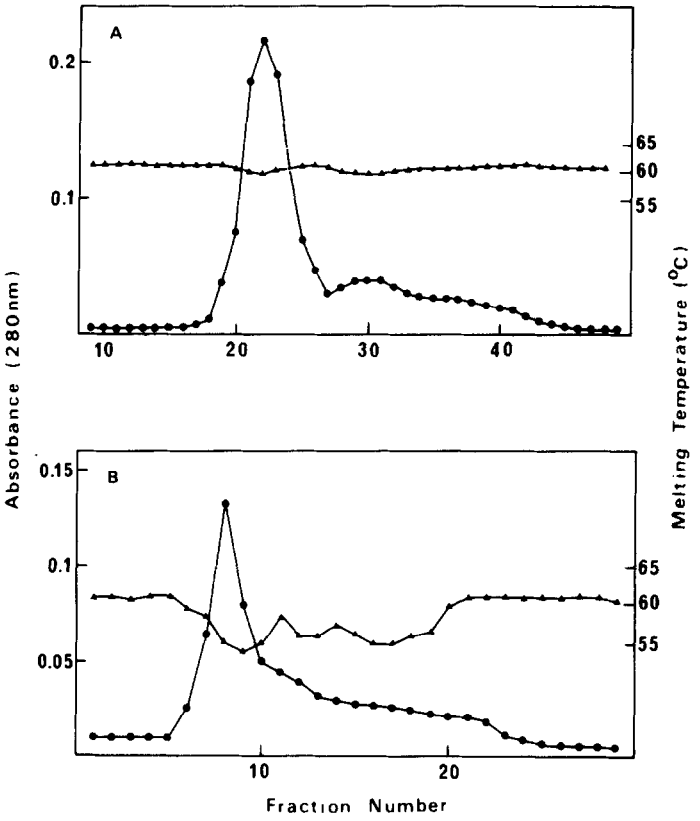

Fig. 3 Sephadex G-200 gel-filtration of the 0.3M KCl fraction from DNA-cellulose chromatography of total cell protein. The 0.3M KCl fraction was concentrated to 5ml by sucrose reduction dialysis before being applied to the column. (o) Protein as determined by absorbance, (Δ) Melting temperature of poly [d(A-T)] with the addition of the appropriate protein fraction.(A) mock-infected cells,(B) virus-infected cells. The gross difference in fraction numbers between A and B was because columns of different dimensions were used.

it (1) binds specifically to single-stranded DNA-cellulose. In our earlier experiments proteins were passed first through a double-stranded DNA-cellulose column connected in series to a single-strand column. Only fractions subsequently eluted from the second column could depress the T_m of poly [d(A-T)] . (2) Depresses the melting temperature of poly [d(A-T)] and (3) Specifically stimulates homologous DNA polymerase.

Our HDP has a mol.wt. of under 30,000 daltons since it passes through Amicon filters UM30 (Table 1). It is presumably, therefore, different from the HDP's reported previously and referred to as HSV-2 infected cell specific polypeptides 11 and 12 (18) and HSV-1 infected cell protein 8 (19),

Table 1

DNA polymerase present in the assay	Protein added	Radioactivity incorporated from [^{3}H] dTTP (counts/20 min)	dTMP residues incorporated in 20 min. (nmoles)
HSV-DNA polymerase	none	435 ± 20	1.65 ± 0.07
wash-through from	I	1122 ± 119	4.25 ± 0.50
DNA-cellulose	II	522 ± 64	1.98 ± 0.23
HSV-DNA polymerase	none	382 ± 94	1.44 ± 0.35
eluted from	I	1491 ± 211	5.65 ± 0.69
DNA-cellulose with 0.4M KCl	II	383 ± 38	1.45 ± 0.14

Stimulation of HSV-DNA polymerase by protein fractions from Sephadex gel-filtration. Fractions 14-20 from the Sephadex G-200 column shown in fig. 3B were pooled and concentrated by Amicon filtration using a UM30 filter. The protein solution which passed through the filter is referred to as protein I in the table, and the remaining solution as protein II. 40 µg of protein from each of these were added to the DNA polymerase assay.

The HSV DNA polymerase refers to the unbound (wash-through) and bound (0.4M KCl fraction) components during the final purification step on single-stranded DNA-cellulose columns. The polymerase in the wash-through fractions was first concentrated on a small DEAE-cellulose column. It did not bind to DNA-cellulose on re-chromatography, whereas over 90% of the polymerase eluted with the KCl did. Results are the means ± S.D. (n=3) after deduction of appropriate background radiation. Neither protein I or II had any DNA polymerase activity by themselves.

since these have molecular weights of about 145 x 10^3. Bayliss et al (5) considered their DNA-binding protein BP9, from HSV-1 infected cells, might be analogous to gene 32 protein by virtue of its preferential binding to single-stranded DNA. This protein has a molecular weight of about 40 x 10^3, closer to that of our protein.

Our work, and that of others (18), shows that the 0.3M KCl fraction from DNA-cellulose contains some of both the virus-induced enzymes DNA polymerase and alkaline DNAase. However, these are separated from our HDP on the Sephadex G-200 by virtue of their higher molecular weights (14,20).

Even if some contaminating nuclease were present in our HDP fraction it would possess only 10% or less of its maximum activity at the pH 7.6 used in the melting assay (20). In any event we detected neither DNA polymerase nor DNAase activity. In addition, when the proteins and poly [d(A-T)] solutions were heated separately to the expected lower melting temperature then the hyperchromic effect occurred immediately on mixing.

The melting activity appears to be rather unstable since on freezing and thawing, or even after a week at $4^{o}C$, a biphasic melting profile is observed, a portion of the poly [d(A-T)] now melting at the normal temperature of $61^{o}C$ (under our conditions).

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