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Demonstration of a stimulatory protein for virus-specified DNA polymerase in phorbol ester-treated Epstein–Barr virus-carrying cells

(Epstein–Barr virus DNA replication protein/inhibition by phosphonoformate/antibodies in sera from patients with nasopharyngeal carcinoma)

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ABSTRACT **A heat-labile Epstein–Barr virus-specific DNA polymerase stimulatory protein having a molecular mass of 45 kDa was purified from phorbol 12-myristate 13-acetate-treated P3HR-1 cells by column chromatography. The virus DNA polymerase stimulatory protein was precipitated by sera from patients with nasopharyngeal carcinoma but not by sera from healthy donors. The interaction of the stimulatory protein with DNA polymerase was stoichiometric. Furthermore, this protein stimulated Epstein–Barr virus DNA polymerase but not herpes simplex virus type 1 or type 2 or human DNA polymerase α. The stimulatory protein did not alter the K_m value of dTTP or DNA but did increase the V_{max} of DNA polymerase. Salt concentrations between 100 mM and 150 mM KCl were optimal for this protein-induced stimulation of Epstein–Barr virus DNA polymerase activity. The presence of the stimulatory protein in the reaction mixture enhanced the sensitivity of virus DNA polymerase to phosphonoformate.**

There are five types of herpesviruses that induce human disease. Among these, Epstein–Barr virus (EBV) is known to have close association with Burkitt lymphoma and nasopharyngeal carcinoma (NPC) (1). The EBV genomes present in both virus-infected and virus-carrying cells (2) are able to induce several virus-specified proteins. It has been shown that EBV induces a virus-specified DNA polymerase and DNase (3–6). The activities of both enzymes increase when EBV-carrying cells, P3HR-1 cells, are treated with the tumor-promoting ester phorbol 12-myristate 13-acetate (PMA) (3, 7). Procedures to highly purify EBV DNA polymerase by various column chromatographic techniques have been described (3, 7–10). Based on our experience, recovery of the enzyme activity is poor and quite inconsistent throughout the purification. This raises the question of whether the loss of enzyme activity during purification is the result of the separation of a stimulatory protein from the enzyme or is due to instability of the enzyme. This communication describes the purification and some properties of an EBV-specific DNA polymerase stimulatory protein.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade or better. Unlabeled dNTPs, calf thymus DNA, dithiothreitol, bovine serum albumin, phenylmethylsulfonyl fluoride, and PMA were obtained from Sigma. ICN was the source of [^{3}H]dTTP. Phosphonoformate was a gift from Astra A.B. (Sodertalje, Sweden). NPC patient sera used for immunostaining were provided by Tseng-Chou Lynn from the National Taiwan University Hospital.

Treatment of P3HR-1 Cells with PMA. The EBV-producer cell line P3HR-1 was maintained at 37°C in humidified 5% CO_2/95% air. The P3HR-1 cells were grown as a stationary-phase suspension culture in RPMI 1640 medium containing 10% fetal calf serum. For the induction of virus-specified proteins, cells seeded at a density of 5×10^5/ml were grown as suspension cultures for 6 days at 34°C. The cells were then pelleted and suspended in fresh medium containing PMA at 40 ng/ml. After a 4-day incubation, the cells were collected by centrifugation and stored at −70°C until use.

Preparation of Viral and Human DNA Polymerases. EBV-specified DNA polymerase from PMA-treated P3HR-1 cells was purified as described (3). This involved DEAE-cellulose, phosphocellulose, and DNA-cellulose column chromatography. The final specific activity of EBV DNA polymerase was $>4.0 \times 10^5$ units (defined below) per mg of protein. Herpes simplex virus (HSV) type 1 and type 2 DNA polymerases were purified from HSV-1 (KOS strain)- and HSV-2 (333 strain)-infected HeLa cells, respectively, as described (11, 12). Human DNA polymerase α was purified from HeLa cells by a published procedure (12).

DNA Polymerase Assay. The reaction mixture for the assay of different types of DNA polymerase was the same except for the concentration of KCl. The mixture (100 μl) was composed of 50 mM Tris·HCl, pH 8.0/4 mM $MgCl_2$/0.5 mM dithiothreitol/0.1 mM dATP/0.1 mM dGTP/0.1 mM dCTP/5 μM [^{3}H]dTTP (40 mCi/μmol; 1 Ci = 37 GBq) containing bovine serum albumin at 0.2 mg/ml, 15 μg of activated calf thymus DNA, and different amounts of KCl. For EBV, HSV-1, HSV-2, and human DNA polymerase α, the concentrations of KCl were 150, 200, 200, and 0 mM, respectively. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of dTMP into activated DNA per min at 37°C as described (12).

Electrophoresis, Blotting, and Immunostaining. NaDodSO$_4$/polyacrylamide gel electrophoresis was carried out according to Laemmli (13) using 10% gels. After electrophoresis, slab gels were silver stained following the method of Merril *et al.* (14). Proteins were transferred to nitrocellulose paper according to the method of Bowen *et al.* (15). Immunostaining of proteins on nitrocellulose paper was carried out according to Legochi and Verma (16).

Serological Test. Immunoprecipitation of EBV DNA polymerase stimulatory protein was accomplished by adding diluted (1:5) NPC patient serum or control serum from healthy individuals. After incubation at room temperature for 20 min, 10 μl of previously swollen protein A-conjugated Sepharose (Pharmacia) was added. After further incubation for 1 hr at 4°C, the immunocomplexes were pelleted by

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Abbreviations: EBV, Epstein–Barr virus; PMA, phorbol 12-myristate 13-acetate; HSV, herpes simplex virus.

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centrifugation and the supernatant was examined for DNA polymerase stimulatory protein activity using purified EBV DNA polymerase.

RESULTS

Demonstration and Purification of EBV DNA Polymerase Stimulatory Protein. A crude homogenate of PMA-treated P3HR-1 cells was passed first through a DEAE-cellulose column to remove nucleic acid and then through a second DEAE column for further purification (3). EBV DNA polymerase having a total apparent activity of 5.7×10^3 units was then loaded onto a phosphocellulose column. This column was developed with a linear gradient of 50–400 mM phosphate buffer that contained 20% glycerol/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/2 mM dithiothreitol. Fractions of 3.4 ml were collected and 10 μl of each was assayed for DNA polymerase activity with and without 0.2 unit of purified EBV DNA polymerase preparation (after DNA-cellulose column chromatography). The results are shown in Fig. 1*A*. The recovery of apparent DNA polymerase (fractions 45–55) was ≈10%. There was a factor in several fractions that eluted ahead of the EBV DNA polymerase activity peak and stimulated added EBV DNA polymerase. Those fractions (tubes 36–39) were pooled and applied to a DNA-cellulose column (0.8 × 2 cm), which was developed with a linear 50–400 mM phosphate gradient. The elution profile for the stimulatory factor is shown in Fig. 1*B*. The peak fractions (tubes 19–21, 2.5-ml fractions) were pooled and applied to a heparin-Sepharose column (0.8 × 1.5 cm). This column was developed with a linear 100–400 mM phosphate gradient. The elution profile for the stimulatory protein from this column is shown in Fig. 1*C*. The concentration of protein in these peak fractions (1.0 ml each) was <1 μg/ml. There was no intrinsic DNA polymerase activity. To assess the purity of the protein(s) in the peak fraction (tube 18), it was examined using $NaDodSO_4$/PAGE followed by

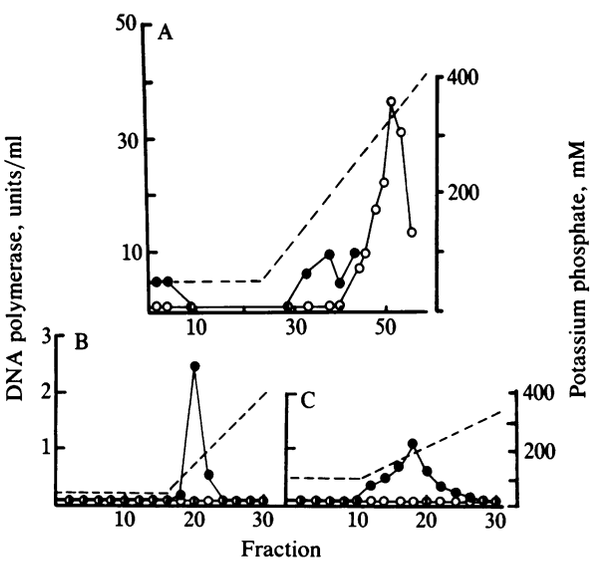

FIG. 1. Column chromatography profiles of the EBV DNA polymerase stimulatory protein. (*A*) The cellular extract (5×10^9 cells) was passed through a DEAE-cellulose column as described (3) and then loaded onto a phosphocellulose column. (*B*) The peak fraction from the phosphocellulose column was loaded onto a single-stranded DNA-cellulose column. (*C*) The peak fraction from the DNA-cellulose column was purified on a heparin-Sepharose column. The amount of EBV DNA polymerase used for detecting the activity of the stimulatory protein was 0.1 unit per assay (●). ○, Intrinsic activity of DNA polymerase in fraction. ---, Potassium phosphate gradient.

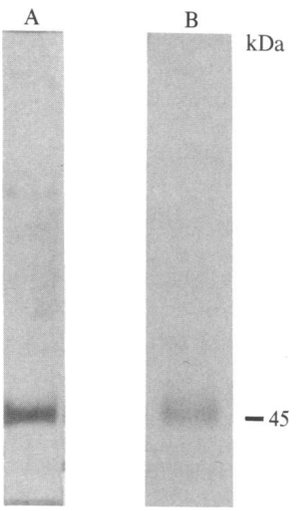

FIG. 2. Electrophoresis profiles of the final preparation of the stimulatory protein. After purification through the heparin-Sepharose column chromatography step, the stimulatory protein was electrophoresed as described (17). Slab gels were either silver stained (lane A) or transferred to nitrocellulose paper and immunostained by a technique using serum from a NPC patient (lane B). The amount of activator shown here caused maximal stimulation of at least 1 unit of EBV DNA polymerase, which is equivalent to 100 μl of the stimulatory protein preparation described in Fig. 3.

silver- or immunostaining (Fig. 2). One major protein band was detected by the silver-staining technique (Fig. 2, lane A). One protein with an apparent molecular mass of 45 kDa was recognized by serum from patients with NPC (Fig. 2, lane B) but not by serum from normal individuals (data not shown). Occasionally, a 98-kDa protein was also immunostained.

Presence of Antibodies Against the Stimulatory Protein in Sera of Patients with Nasopharyngeal Carcinoma. To further substantiate that the protein band that could be stained on nitrocellulose paper was the stimulatory protein, the stimulatory protein was mixed with sera from NPC patients or from normal individuals for 20 min at room temperature. To each protein A-conjugated Sepharose was added as described. Immunocomplexes were pelleted by centrifugation after 1 hr of incubation at 4°C. The supernatant was assayed for the presence of this protein and the results are shown in Table 1. The stimulatory protein was immunoprecipitated by sera from NPC patients but not by sera from normal individuals. Since the sera from NPC patients stained only one protein on the nitrocellulose sheet after immunological blotting, the protein band observed in Fig. 2 (lane B) with a molecular mass of 45 kDa could be the stimulatory protein.

Interaction of the Stimulatory Protein and DNA Polymerase. Different amounts of the stimulatory protein were added to a fixed amount (0.2 unit) of EBV DNA polymerase. The degree of activation reached a plateau with a maximum degree of activation of about 8-fold (Fig. 3*B*). This varied depending on the degree of contamination of the protein in the DNA polymerase preparation used. With a fixed amount of the stimulatory protein, different amounts of enzyme were added, and a marked stimulation was observed at a low concentration of enzyme (<0.6 unit). No further stimulation was observed when more enzyme was added (Fig. 3*A*). Thus, the results shown in Fig. 3 indicate a stoichiometric interaction of activator and DNA polymerase.

The specificity of the stimulatory protein toward various types of DNA polymerase was examined, and the results are shown in Table 2. The amount of activator that gave a 4-fold stimulation of EBV DNA polymerase did not stimulate HSV-1, HSV-2, or human DNA polymerase α.

Table 1. Interaction of the stimulatory protein with sera of nasopharyngeal carcinoma patients or normal individuals

Stimulatory protein	Sera added	cpm
−	—	384
+	—	1123
+	NPC	262–356
+	Normal	998–1453

The stimulatory protein (the amount capable of fully stimulating 0.01 unit of EBV DNA polymerase) was incubated with an equal volume of diluted (1:5) NPC patient sera or normal healthy individual sera for 20 min at room temperature and then added to 10 μl of previously swollen protein A-Sepharose and incubated for 1 hr at 4°C. The mixture was centrifuged, and 10 μl of supernatant was assayed for the remaining stimulatory protein in (total volume, 0.1 ml) 150 mM KCl/50 mM Tris·HCl, pH 8.0/4 mM $MgCl_2$/0.5 mM dithiothreitol/0.1 mM dATP/0.1 mM dCTP/0.1 mM dGTP/5 μM [^{3}H]dTTP (40 μCi/ml) containing bovine serum albumin at 0.2 mg/ml, 15 μg of activated calf thymus DNA, and 0.01 unit of EBV DNA polymerase. After 20 min of incubation at 37°C, 50-μl aliquots were spotted onto GF/A filters and the filters were washed with cold 5% trichloroacetic acid and 1 mM sodium pyrophosphate to determine acid-insoluble radioactivity. Results presented are ranges of values obtained with six individual sera. The stimulatory protein control was treated in the same fashion except that phosphate-buffered saline was used in place of sera in the first incubation.

Effect of the Stimulatory Protein on the Kinetic Behavior of DNA Polymerase. The effect of the stimulatory protein on the K_m and V_{max} of EBV DNA polymerase was examined, and typical results are shown in Fig. 4. This protein did not alter the K_m of the enzyme for dTTP or DNA but it did increase the V_{max} of DNA polymerase.

Effect on the Behavior of DNA Polymerase Toward Salt and Phosphonoformate. Salt is known to stimulate EBV DNA polymerase with an optimal concentration between 100 mM and 150 mM KCl. Addition of the stimulatory protein did not alter the profile in a qualitative sense but did alter it in a quantitative sense (Fig. 5*A*). When the effect of phosphonoformate on EBV DNA polymerase was tested in the presence and absence of the amount of stimulatory protein that gave maximum stimulation, the stimulatory protein enhanced the sensitivity of the polymerase (Fig. 5*B*).

Heat Stability of the Activator. Heat inactivation of the EBV DNA polymerase stimulatory protein is shown in Fig. 6. The stimulatory effect of this protein on EBV DNA polymerase was decreased by 60% after incubation for 10 min at 65°C. Further incubation resulted in continued inactivation of the stimulatory protein.

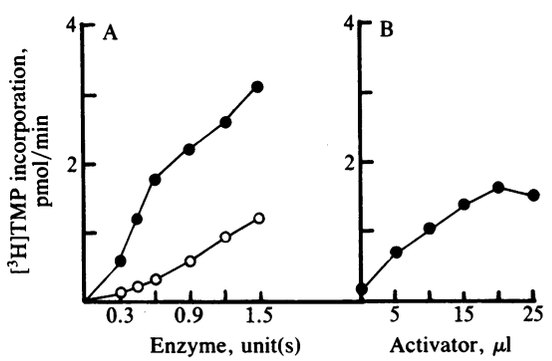

FIG. 3. Interaction of the stimulatory protein and EBV DNA polymerase. Reactions were carried out with a variable amount of DNA polymerase and either a fixed amount (2.5 μl) of the stimulatory protein (●) or no stimulatory protein (○) (*A*) or with a fixed amount (0.2 unit) of EBV DNA polymerase and a variable amount of the stimulatory protein (*B*).

Table 2. Specificity of the stimulatory protein toward DNA polymerase

DNA polymerase	cpm	
	Without SP	With SP
Human α	1305	1197
HSV-1	518	594
HSV-2	476	368
EBV	464	2029

Ten microliters of the stimulatory protein (SP) described in Fig. 3 was used per assay mixture.

DISCUSSION

A specific EBV DNA polymerase stimulatory protein having an apparent molecular mass of 45 kDa has been found. This protein could be separated from EBV DNA polymerase by phosphocellulose column chromatography. This may at least partly explain why the yield of the apparent EBV DNA polymerase activity through this purification step varies from one preparation to another. The interaction of EBV DNA polymerase and its stimulatory protein is apparently stoichiometric. Upon PMA induction of EBV DNA polymerase activity in P3HR-1 cells, the relative amounts of the stimulatory protein and EBV DNA polymerase did not change. We have not fractionated a homogenate from uninduced cells but we did add a purified polymerase (the preparation after the DNA-cellulose step) to such a homogenate and observed only additive activity. Similarly, addition of purified stimulatory protein did not increase the activity of the homogenate from uninduced cells. Concurrent induction of the stimulatory protein and polymerase is indicated. It is possible that this protein is an integral part of EBV DNA polymerase and that the catalytic portion of the enzyme becomes dissociated from the stimulatory protein or proteolysis during the purification separates these proteins. This will require further investigation. It can be concluded that the interaction of this protein with EBV DNA polymerase is quite specific. It should be stated that, in the purified stimulatory protein preparation, there was no detectable DNA topisomerase or DNA endo- or exonuclease activity using the assay procedure described in refs. 18–20. The

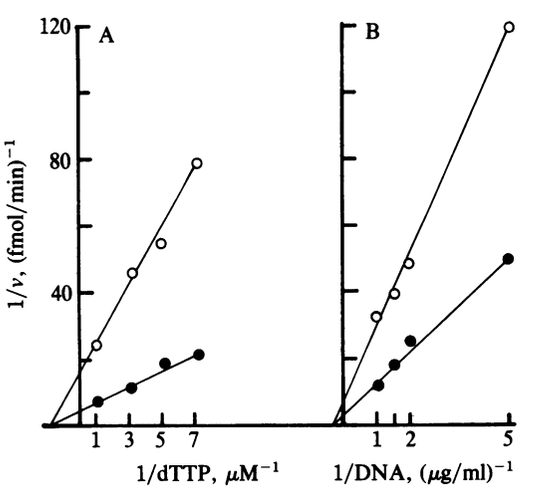

FIG. 4. Effect of the stimulatory protein on the kinetic properties of EBV DNA polymerase. Data for Lineweaver–Burk plots were obtained by varying the concentration of either [^{3}H]dTTP (*A*) or activated calf thymus DNA (*B*) with (●) or without (○) addition of the stimulatory protein (5 μl as described in Fig. 2) and using 0.2 unit of EBV DNA polymerase. The reaction time was 20 min at 37°C.

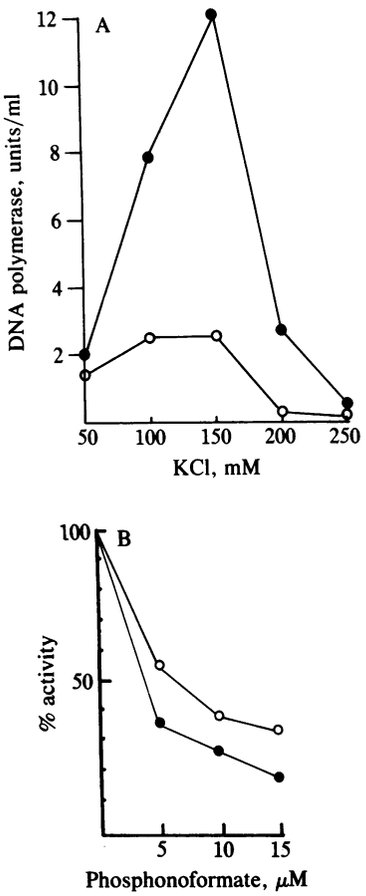

FIG. 5. Effect of the stimulatory protein on the salt (*A*) and phosphonoformate (*B*) sensitivities of EBV DNA polymerase. Studies were performed with (●) or without (○) added stimulatory protein, which caused a 6-fold activation of 0.1 unit of EBV DNA polymerase and was equivalent to 5 μl of the preparation described in Fig. 3. The amount of EBV DNA polymerase used was 0.2 unit. The KCl concentration used in *B* was 150 mM.

detailed mechanism of DNA polymerase activation by this protein is still unclear.

The appearance of two peaks of EBV DNA polymerase activity in the final purification step (DNA-cellulose column chromatography) has been reported (3). These two peaks of activity were stimulated by salt to different extents and had different sensitivities to phosphonoformate, a potent DNA polymerase inhibitor. In this work, by careful separation of the stimulatory protein from DNA polymerase on a phosphocellulose column, only one peak of DNA polymerase was observed in the final purification step. In this communication we have reported that stimulatory protein alters the KCl concentration dependence and the phosphonoformate sensitivity of EBV DNA polymerase. It is likely that the observation of two peaks of activity at the DNA-cellulose column step was due to the presence of the stimulatory protein in one of those peaks. This may also provide an explanation for the differences in the properties of EBV DNA polymerase observed by different investigators.

A higher titer of antibodies against EBV DNase was found in sera from patients with NPC than in sera from normal individuals (21, 22). Antibodies against the EBV DNA polymerase stimulatory protein were also found in sera from

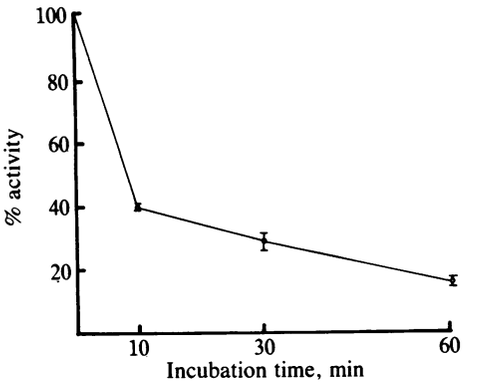

FIG. 6. Stability of the stimulatory protein at 65°C. Purified stimulatory protein was incubated at 65°C in 100 mM potassium phosphate (pH 7.5) for the times indicated. The stimulatory protein was assayed by examining its ability to stimulate DNA polymerase (0.2 unit). The protein initially used (5 μl as described in Fig. 3) could not stimulate more than 0.2 unit of DNA polymerase.

patients with NPC and not in sera from normal individuals. Whether sera from patients with NPC have a higher frequency and higher titer of antibodies against EBV DNA polymerase activator than sera from normal individuals is not known.

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