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Association of Epstein-Barr Virus Early Antigen Diffuse Component and Virus-Specified DNA Polymerase Activity

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The role of Epstein-Barr virus (EBV) early antigen diffuse component (EA-D) and its relationship with EBV DNA polymerase in EBV genome-carrying cells are unclear. EBV-specified DNA polymerase was purified in a sequential manner from Raji cells treated with phorbol-12,13-dibutyrate and *n*-butyrate by phosphocellulose, DEAE-cellulose, double-stranded DNA-cellulose, and blue Sepharose column chromatography. Four polypeptides with molecular masses of 110,000, 100,000, 55,000, and 49,000 daltons were found to be associated with EBV-specified DNA polymerase activity. A monoclonal antibody which could neutralize the EBV DNA polymerase activity was prepared and found to recognize 55,000- and 49,000-dalton polypeptides. An EA-D monoclonal antibody, R3 (G. R. Pearson, V. Vorman, B. Chase, T. Sculley, M. Hummel, and E. Kieff, J. Virol. 47:183–201, 1983), was also able to recognize these same two polypeptides associated with EBV DNA polymerase activity. It was concluded that EBV EA-D polypeptides, as identified by R3 monoclonal antibody, are critical components of EBV DNA polymerase.

Epstein-Barr virus (EBV) is a herpesvirus which is closely associated with some human malignancies, such as Burkitt's lymphoma and nasopharyngeal carcinoma (NPC). In addition, it is associated with some human infectious diseases. One of the critical enzymes responsible for EBV DNA replication in productively infected cells is EBV-specified DNA polymerase (DP). With a partially purified preparation of this enzyme, it was found that DP activity could be stimulated by salts, such as potassium chloride and ammonium sulfate, and inhibited by phosphonoformate (1, 2, 5, 11, 17). Furthermore, the substrate specificity of EBV DP is different from those of human cellular DPs. In the course of EBV DP purification from phorbol-12-myristate-13-acetate-treated P3HR-1 cells, an EBV DP-specific stimulatory protein with a molecular mass of 45,000 daltons was found, and an antibody against this stimulatory protein could be demonstrated in serum from patients with NPC. The stimulatory protein did not alter the K_m value of the substrate but did increase the V_{max} value of highly purified EBV DP (1). It was reported that the major protein component in EBV DP from *n*-butyrate-treated P3HR-1 cells is a polypeptide having a molecular weight of 110,000 (7). This is quite different from the DP induced by herpes simplex virus, which is composed of two polypeptides with different molecular weights (14).

Several viral proteins are synthesized during the early period of EBV replication. Based on resistance to methanol and cellular localization by immunofluorescence assay, two early antigens, diffused (EA-D) and restricted, were classified (6). Monoclonal antibodies (R3) against EA-D were prepared and found to recognize two virus-induced polypeptides with molecular weights of 50,000 and 52,000 (12). The gene responsible for the synthesis of these two polypeptides was found to be in the *Bam*HI M fragment of the EBV genome (18). The function of EA-D polypeptides is presently unclear. In this study, we suggested that EBV DP has EA-D polypeptides as its components.

EBV DP is induced by chemical agents not only in virus producer P3HR-1 cells, but also in latently infected cells such as Raji cells (2, 7, 8, 10, 11, 17). The general properties of EBV DP induced in these cell lines by different conditions are similar. High titers of antibody against EA-D were found in NPC serum (6, 12), and recently, antibodies which could neutralize the activity of a relatively crude preparation of EBV DP were also found (17). The neutralizing activity could be the result of antibody interaction with either EBV DP-specific stimulatory protein or other components of EBV DP. To induce enough activity to further characterize EBV DP, we chose Raji cells treated with phorbol-12,13-dibutyrate and *n*-butyrate as the source of the enzyme. Sequential chromatography on phosphocellulose, DEAE-cellulose, double-stranded DNA-cellulose, and blue Sepharose columns with salt-gradient elution was employed for purification.

All the buffers used in chromatography procedures contained 20% glycerol, 5 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml each of pepstatin and leupeptin; salt concentrations and pH values are indicated where appropriate. The linear gradients used for column elution contained 0 to 0.6 M KCl in 50 mM KPO_4 buffer, pH 8.0, except for that used for DEAE-cellulose chromatography, which contained 50 mM to 0.4 M KPO_4, pH 7.5. Raji cells were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 μg of kanamycin per ml. The chemical inducers were added at final concentrations of 50 nM for phorbol-12,13-dibutyrate and 4 mM for *n*-butyrate. The cells were harvested at 48 h postinduction, washed twice with cold phosphate-buffered saline, and suspended in extraction buffer containing 0.3 M KCl, 40 mM Tris hydrochloride (pH 7.5), 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol. The cells were then freeze-thawed three times, and the extract was centrifuged at 12,000 × *g* for 15 min. The supernatant was passed over a DEAE-cellulose column that had been preequilibrated with 0.4 M KPO_4

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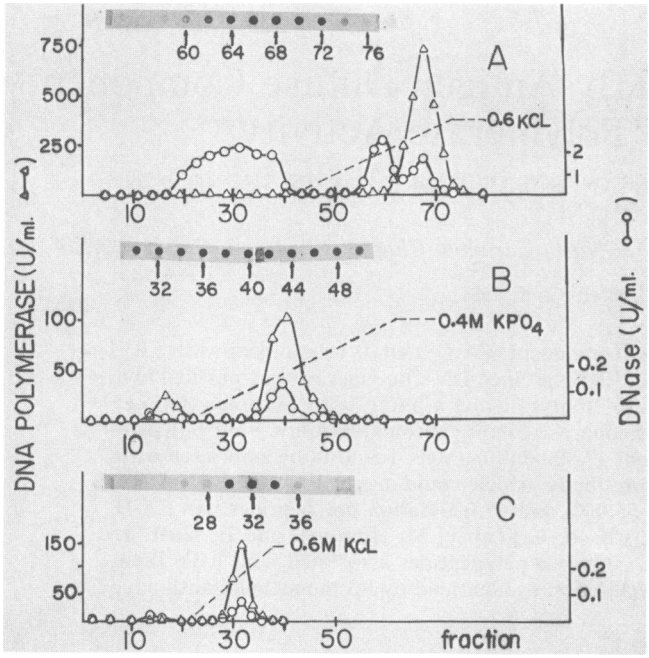

FIG. 1. Purification profiles of EBV DP on phosphocellulose column (A), DEAE-cellulose column (B), and double-stranded DNA-cellulose column (C). Immunodot results are shown in the insets; the numbers represent fractions.

buffer, pH 7.5, to remove nucleic acids. The EBV DP was then purified in four chromatography steps (Fig. 1 and 2A), and EBV DP and DNase activities were monitored. The assay mixture for EBV DP was composed of 50 mM Tris hydrochloride (pH 8.0), 4 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.2 mg of bovine serum albumin per ml, 100 μM each of

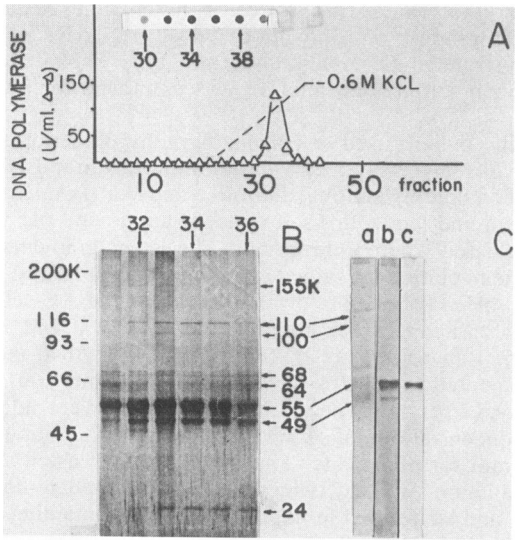

FIG. 2. (A) Purification profile of EBV DP on blue Sepharose column. Immunodot result is shown in the inset; the numbers represent fractions. (B) Silver stain using fractions 31 to 36 after blue Sepharose column. Molecular weight of proteins (in thousands [K]) is indicated. (C) Western blot immunostain. Primary antibodies used were pooled NPC sera (lane a), monoclonal antibody R3 (lane b), and MCAb 49-6 (lane c).

TABLE 1. Purification of EBV DP

Purification step	Total activity (U)	Recovery (%)	Sp act (U/mg)	Purification (fold)
Crude extract	60,107	100	402	1
Phosphocellulose	35,431	60	4,724	11.8
DEAE-cellulose	2,035	3.4	4,915	12.2
Double-stranded DNA-cellulose	1,038	1.7	13,308	33.5
Blue Sepharose	642	1.1	89,167	223

dATP, dCTP, and dGTP, 5 μM [^{3}H]TTP, and 100 mM $(NH_4)_2SO_4$ in a total volume of 100 μl. One unit of EBV DP activity is the same as that defined previously (1). The EBV DNase assay was essentially the same as that reported previously (16).

EBV DP was virtually separated from EBV DNase activity by the first chromatographic step (phosphocellulose). The majority of the EBV DP activity was lost after the DEAE-cellulose column step. This could be explained by the dissociation of EBV DP-specific stimulatory protein from EBV DP at this stage of the purification. When the stimulatory protein was added back to the DP activity peak from DEAE-cellulose, DP activity increased more than sevenfold (data not shown). EBV DP was purified more than 200-fold by the four chromatographic steps (Table 1). If the sevenfold stimulation of EBV DP by the stimulatory protein is taken into account, then the EBV DP was purified more than 1,000-fold by this entire purification protocol.

To check the purity of the EBV DP, fractions 31 to 36 from the final step in the purification were electrophoresed on a sodium dodecyl sulfate–7.5% polyacrylamide gel. Silver staining showed that four polypeptides having molecular weights of 110,000, 100,000, 55,000, and 49,000 were associated with the DP activity peak (Fig. 2B). The nature of 68,000-, 64,000-, and 24,000-dalton polypeptides is unclear at present and is under further investigation. Pooled sera from patients with NPC recognized all four polypeptides (Fig. 2C, lane a). A monoclonal antibody, designated MCAb 49-6, which could neutralize EBV DP activity, was prepared in this laboratory with a partially purified enzyme preparation as an antigen. This antibody could recognize two polypeptides with molecular masses of 55,000 and 49,000 daltons in peak DP fractions from the blue Sepharose column (Fig. 2C, lane c). Since these two polypeptides have a molecular mass similar to that reported previously for EA-D (9, 13), this observation raised the possibility that the two polypeptides detected by MCAb 49-6 were EA-D. A monoclonal antibody (R3) against EA-D was examined for its ability to recognize those two polypeptides in our preparation. This antibody recognized the same two polypeptides that MCAb 49-6 did (Fig. 2C, lane b). To examine whether EBV DP was always associated with EA-D, dot blots of fractions throughout the purification were immunostained with monoclonal antibody R3 (Fig. 1 and 2A). The results showed that EBV DP could not be separated from EA-D on phosphocellulose, double-stranded DNA-cellulose, and blue Sepharose columns, and the intensity of staining correlated with the peaks of EBV DP activity. At the DEAE-cellulose step, a component(s) of EA-D may be partially separated; however, this was not investigated further. The same pattern was obtained with MCAb 49-6 (data not shown).

The MCAb 49-6 was further purified on a protein A-Sepharose column (4) and was determined to be of the immunoglobulin G 2a subtype, based on the elution condition from the column. Its ability and specificity to neutralize EBV DP

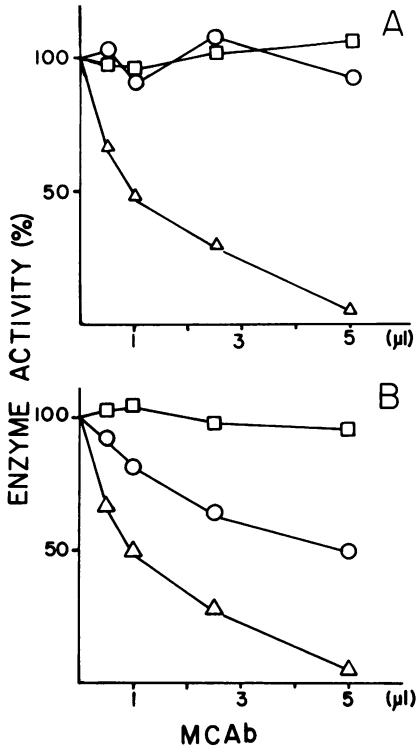

FIG. 3. (A) Neutralization with MCAb 49-6 on EBV DP (△), herpes simplex virus type 2 DP (○), and human DP α (□). (B) Neutralization with MCAb 49-6 (△), R3 (○), and monoclonal antibody against EBV early antigen restricted component (□) on EBV DP purified on blue Sepharose column. The enzyme activity was 0.3 to 0.5 U for each assay.

activity were examined. EBV, herpes simplex virus type 2, and human α DPs were incubated with MCAb 49-6 at room temperature for 20 min, and then their respective reaction mixtures were added to initiate the enzyme reaction at 37°C for 20 min. The results are depicted in Fig. 3. MCAb 49-6 strongly neutralized EBV DP activity but had no effect on herpes simplex virus type 2 and human DPs (Fig. 3A). The monoclonal antibody (R3) against EA-D also neutralized EBV DP activity. The monoclonal antibody against another early antigen, early antigen restricted component, did not affect the enzyme activity (Fig. 3B). EBV DP derived from P3HR-1 cells, an EBV producer, was also neutralized by the R3 and MCAb 49-6 monoclonal antibodies (data not shown).

In this study, EBV-specified DP was purified from Raji cells induced with phorbol-12,13-dibutyrate and *n*-butyrate, and four polypeptides having molecular masses of 110,000, 100,000, 55,000, and 49,000 daltons coincided with EBV DP activity and were recognized by NPC serum. Thus, the neutralizing activity of NPC serum could be the result of the interaction with these polypeptides, in addition to EBV DP-specific stimulatory protein. The polypeptide of 110,000 daltons appears to be a component of EBV DP, which is in agreement with results of other investigators (7). However, the 110,000-dalton polypeptide is insufficient for EBV DP activity, according to the results presented here. The polypeptides with molecular masses of 55,000 and 49,000 daltons are essential to EBV DP activity and appear to be components of EA-D (9). It is suggested that EA-D is a critical component of EBV DP activity and may therefore function in EBV DNA replication. The observations made by others (3, 11, 15) also substantiate this notion.

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